AD_____

GRANT NUMBER: DAMD17-94-J-4476

TITLE: Network Interaction of erbB, neu-erbB2, erbB3, erbB4, in the Biological Response to NDF/neuregulins

PRINCIPAL INVESTIGATOR: Dr. David F. Stern

CONTRACTING ORGANIZATION: Yale University School of Medicine
New Haven, Connecticut 06520-8047

REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Annual 28 Sep 94 - 27 Sep 95

4. TITLE AND SUBTITLE
Network Interaction of erbB, neu-erbB2, erbB3, erbB4, in the Biological Response to NDF/neuregulins

5. FUNDING NUMBERS
DAMD17-94-J-4476

6. AUTHOR(S)
Dr. David F. Stern

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University School of Medicine
New Haven, Connecticut 06520-8047

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The gene encoding the neu/erB-2/HER-2 receptor tyrosine kinase is one of a small number of genes known to be altered in human breast and ovarian carcinoma. It is amplified and/or over expressed in approximately one-fourth of these cancers. With its probable role in etiology, and expression at the cell surface, neu has great potential as a prognostic indicator and therapeutic target. Efforts to exploit this receptor in the clinical arena have been impeded by the failure to consider regulation of neu by hormones and co-receptors. This has been complicated by the fact that at least seven different peptide hormones, epidermal growth factor (EGF), transforming growth factor-alpha, amphiregulin, betacellulin, heparin-binding EGF-like growth factor, epiregulin, and the diverse neuregulins are all able to activate neu. They work by binding to the co-receptors EGF receptor, erB-3 and erB-4, which then dimerize with neu. Work by this laboratory has shown that each one of these factors activates a different constellation of receptors, leading to a great diversity of possible responses. Moreover, the coupling of cellular responses to each factor is determined by the subset of receptors expressed in each tissue. Thus in order to interpret the function of any one of the factors or receptors, including neu, it is essential to consider the influences provided by the entire set.

14. SUBJECT TERMS			15. NUMBER OF PAGES
breast cancer	neu/erB-2/HER-2	EGF receptor	66
NDF/neuregulins	oncogene	growth factor	16. PRICE CODE
receptor tyrosine kinase			

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	**PR**	- Project
G	- Grant	**TA**	- Task
PE	- Program Element	**WU**	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

DOD - See DoDD 5230.24, "Distribution Statements on Technical Documents."
DOE - See authorities.
NASA - See Handbook NHB 2200.2.
NTIS - Leave blank.

Block 12b. Distribution Code.

DOD - Leave blank.
DOE - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
NASA - Leave blank.
NTIS - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

*U.S.GPO:1993-0-358-779

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

✓ Where copyrighted material is quoted, permission has been obtained to use such material.

✓ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

[signature] 10/25/95

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Breast cancer is one of the most important cancers afflicting women and presents challenging treatment decisions. After p53 alterations, the most frequent change in an identified gene is amplification and/or overexpression of the *neu/erbB-2/HER-2* gene, which occurs in up to one-third of breast cancers. The gene product (denoted p185 or non-italicized neu), is a receptor tyrosine kinase (RTK) [1, 2, 3]. This gene was originally discovered in mutant form in chemically-induced rat nervous system tumors and is now known to be a member of the Type 1, or epidermal growth factor (EGF) receptor gene (*erbB*) family. The family includes four receptors, which will be referred to herein as the EGFR, neu, erbB-3, and erbB-4. (See our review, [4], Appendix). Small-scale screens for *neu* alterations in human tumors led to discovery of changes in a number of adenocarcinomas including breast [5], ovarian, gastric, bladder, lung, and colon. Two influential studies showed that *neu*, and not a panel of other oncogenes is amplified in breast and ovarian carcinomas and that this amplification correlates with RNA and p185 overexpression [6, 7]. Numerous studies of *neu* in mammary carcinoma have led to the following conclusions [4] (Appendix).

1.*neu* is amplified in 20-30% of mammary carcinomas, with the frequency of amplification higher in tumors from patients with affected lymph nodes [8, 9, 10, 11, 12, 13, 14, 15, 16]. The gene amplification suggests that there is a selection in the tumors for *neu* overexpression. (It cannot be absolutely ruled out that neighboring genes are selected for amplification.)

2.Amplification correlates well with concomitant RNA and p185 expression [6, 7]. An additional 5% of specimens overexpress the receptor without obvious changes in gene structure or copy number [5, 11, 15, 17].

3.There is no evidence for structural mutations in p185 in human tumors. This negative result is weak owing to the high copy number of genes and the large size of the mRNA. Recent work in the mouse transgenic system suggests that this issue should be reconsidered [18].

3.Amplification and/or p185 overexpression can be found in all grades and stages of carcinomas, but not hyperplasia or dysplasia. It is found more frequently in ductal carcinoma in situ (DCIS) than in infiltrating ductal carcinoma (IDC)[14, 16, 19, 20].

4.Amplification and/or p185 overexpression is associated with poor prognosis, especially in node-positive patients. However the extent of this association and independence from other prognostic markers varies greatly among different studies (reviewed, [4].

Taken together, these data indicate that *neu* amplification and overexpression play a major role in mammary carcinogenesis. This is consistent with findings in model systems: i) the mutated rat *neu* oncogene is as potent as any including *ras* in tissue culture systems. ii)In contrast to other growth factor receptors including the EGFR, overexpression of p185 in the absence of ligand is sufficient to transform cells [21, 22]. iii)Transgenic mice harboring a mutationally activated *neu* oncogene develop multi-focal mammary carcinoma when expressed under control of a murine mammary tumor virus (MMTV) promoter, which confers high level expression in mammary gland and a few other tissues (not found in all studies [23, 24]. iv)Perhaps most compelling is the fact that transgenic mice carrying a structurally normal *neu* gene driven by the MMTV promoter develop metastatic mammary carcinomas [25]. This is noteworthy because it reconstructs what appears to be occurring in human cancer: overexpression of normal p185 in mammary tissue.

Since p185^{neu} is a cell surface protein that seems to play a causal role in mammary carcinogenesis, it is under intensive investigation as a therapeutic target [4, 26]. Phase II clinical trials, in which patients were infused with anti-neu antibody 4D5 have been completed with a roughly 15% response rate, and represent the vanguard for expanded therapeutic trials targetting this receptor (J. Baselga, personal communication). In spite of the findings linking *neu* to mammary carcinoma, and despite the fact that patients are already being exposed to neu antagonists, little is known about the function of neu either in the organism, or in breast cancer.

The physiological function of p185^{neu}, like any hormone receptor, can only be understood in the context of the hormones that regulate it. The EGFR is activated by binding of at least six different peptide hormones, EGF, TGF-α, amphiregulin (AR), betacellulin (βC), epiregulin [27](epi) and heparin-binding EGF-like growth factor (Hb-EGF)[28, 29, 30, 31]. p185, by itself, cannot bind or be activated by these hormones (epi has not been tested). We discovered that EGF and TGF-α, which do not bind to p185, activate p185 Tyr phosphorylation and stimulate p185-associated kinase activity [2, 32]. This phenomenon, now termed **transmodulation**, is dependent upon the co-expression of the EGF receptor with p185 [33, 34, 35, 36]. It probably occurs at least in part through formation of receptor heterodimers [37, 38]. Transmodulation of neu by the EGFR is biologically relevant since it works with EGF, TGF-α, betacellulin (see below) and AR, stoichiometrically activates p185 [32], permits association of substrates [39], and correlates with *in vivo* synergy in transforming ability of these two receptors [40]. Thus wherever the two receptors are co-expressed, EGFr agonists activate *neu* . In cell lines that express both receptors, EGF-regulated neu signaling is at least as important as signaling by the EGFR [41]. Since p185 and the EGFR have distinguishable signaling activities [42], this means that

regulation of neu production provides a means to alter the signal coupled to EGF.

The transmodulation of neu by the EGFR is a prototype for other interactions within the Type 1 receptor family discovered more recently. Ignorance of these interactions has confused many groups studying the EGFR and resulted in a rather muddy literature which is just now being rationalized [4, 43]. For example, several laboratories independently identified an activity termed Heregulin, neu diffentiation factor, gp30, p75, neuregulin, ARIA, and Glial Cell Growth Factor (GGF) [44, 45, 46, 47, 48, 49, 50, 51], a family of related proteins evidently produced by alternate splicing [50](They will be referred to collectively here as NRG, for the composite name neuregulin, or as NDF). At first the NRGs seemed to be neu ligands since they activate p185 tyrosine phosphorylation in the absence of the EGFR and could be cross-linked to neu [45, 49]. However, it is now known that NRGs bind to both erbB-3 and erbB-4 which can then activate neu by transmodulation [52, 53]. A further complication of this receptor system is that erbB-3 lacks robust kinase activity, and itself requires a second receptor for activity [54].

Additional candidates for neu ligands have been identified but not yet expressed in recombinant form and tested for activity [55, 56]. Nonetheless, the independent purification of NRGs by three different laboratories seeking the *neu* ligand suggests that in mammary epithelia the significant inputs to *neu* may come through transmodulation: transmodulating agonists TGF-α, AR, and NDFs are often produced in mammary tissue or cell lines [57, 58, 59] as are the cooperating receptors. Even if these hormones are uniquely responsible for neu activation in mammary tissue, the biological complexities may be enormous. TGF-α and AR, although both EGFR agonists, have somewhat different biological activities [28]. NRGs at first seemed to have radically different activities than EGF

agonists since they promote differentiation in some cell lines [49, 60] (but not others [45, 50]), but this has still not been verified in tissue[4].

In summary, *neu* amplification and overexpression is likely to play a significant role in carcinomas where it occurs. However, the presence of activating mutations and agonistic peptide hormones will regulate *neu* function much more strongly than abundance. The Type 1 RTKs comprise a network in which the signaling potential of each receptor is conditioned not only by the presence of hormones, but is further regulated by the co-expression of related RTKs. The long-term focus of this grant is to define the capabilities of the erbBfamily receptor network: the spectrum of hormones that activate each receptor and receptor combination, and the differences among signalling pathways governed by these receptor systems. These objectives include:

Aim 1: Signalling of individual receptors and receptor combinations will be compared by investigating receptor phosphoryaltions and substrate phosphorylations to determine how receptor interactions modulate signalling specificity.

Aim 2: Functions of NRG ecto- and endo-domains will be analyzed.

Aim 3: Biological activity of NRGs and NRG/TGF-a combinations will be determined in tissue culture.

Cell Line	NRG		EGF		β-cellulin		AR, HbEGF, and TGF-α[c]
	Tyr Phos of IP'd Receptor a	IL-3-independent growth[b]	Tyr Phos of IP'd Receptor	IL-3-independent growth	Tyr Phos of IP'd Receptor	IL-3-independent growth	
LXSN only	NA	N	NA	N	NA	N	NA
EGFR	-	N	+	S	+	S	+
neu	(+)	S	-	N	-	N	-
erbB-3	-	N	-	NT	-	N	-
erbB-4	+	N	-	NT	+	N	-
EGFR + neu	* *	S	+ +	P	+ +	P	+ +
EGFR + erbB-3	+ +	N	+ +	S	+ +	S	+ +
EGFR + erbB-4	+ +	P	+ +	P	+ +	P	+ +
neu + erbB-3	+ +	S	- -	NT	- -	N	- -
neu + erbB-4	+ +	S	- -	N	+ +	S	- -
erbB-3 + erbB-4	+ +	N	- -	NT	- +	N	- -

Table 1. Response of Ba/F3 cell lines to growth factors. (Details [61, 62], **Appendix** and unpublished). [a]- and + refer to stimulation above basal PTyr. *ambiguous with high basal PTyr. NA, not applicable; NT, not tested. [b]N-no survival; S, survival, P, proliferation in the presence of the factor designated. [c]The Tyr phosphorylation pattern induced by these three factors was identical; growth responses were not determined.

I. erbB receptor signalling network.

The foremost objective for year 1 was to determine the ability of each receptor and receptor combination to respond to each of the EGF family agonists. *Without knowing which hormones activate which receptors, it is impossible to interpret the biological function of either.* We undertook a parallel analysis of the aggregate signaling potential of this receptor family by expressing all four human erbB family receptors, singly and in each pairwise combination in a uniform cell background. We have used the resulting cell lines for the first comprehensive evaluation of erbB family receptor activation and coupling to cellular responses by NRG, EGF, Betacellulin, AR, TGF-a, and HB-EGF. (Table 1, and [61, 62]Appendix). This work has resulted in the two publications accompanying in the **Appendix**, and a third manuscript describing the AR and HB-EGF results is in preparation. Completion of this work represents a major step in carrying through the first Aim. It represents completion of Task1, a., b., and c. in the Statement of Work. This task is slightly ahead of schedule. Task 1d. and Task 2 will be performed as proposed (2a. was to have been performed year 1.)

A. Approach. The mouse Ba/F3 pro-B-lymphocyte cell line was chosen for expression of erbB family members because the survival and proliferation of the cells are tightly regulated by exogenous growth factor (IL-3). Prior to the onset of this grant we began to produce cell lines by transfection of all four erbB family cDNAs singly and in each of the pairwise combinations. Production and screening for receptor expression of these cell lines was completed in year 1 of this grant. Each of the cell lines was then stimulated with every EGF-like growth factor, with the sole of exception of epiregulin, which was only discovered this year. We are currently negotiating to obtain epiregulin. The response of cells to these factors was measured in two ways: i.) the Tyr phosphorylation of the receptor(s) expressed in the cell lines was determined by immunoprecipation and

anti-PTyr immunoblotting. This results defines the ability of each factor to couple to each receptor, and in essence defines for the first time the full capabilities of the erbB family factor and receptor network. ii.)The ability of the ligand-activated receptors to enable IL-3-independent survival and proliferation of these lines was determined. These data reveal the degree to which combinatorial interactions govern the growth responses. A brief summary follows, with greater depth provided in the Appendix. Data are summarize in Table 1.

B.Single expressing cell lines. NRG stimulated Tyr phosphorylation of erbB-4 but neither the EGFR nor erbB-3. erbB-3 binds NRG, but does not become Tyr phosphorylated owing to its weak intrinsic kinase activity [54]. The ability of neu on its own to respond to NRG probably occurred through transmodulation via the limiting amount of endogenous erbB-3. The β-cellulin results are noteworthy in demonstrating for the first time that a single growth factor can activate both the EGFR and an additional receptor. Each of these three factors activates a unique set of single receptors: β-cellulin activates the EGFR and erbB-4 equally well, whereas EGF is specific to the EGFR, and NRG is specific to erbB-3 and -4.

C. Transmodulation in double expressor cell lines. This work showed for the first time the unanticipated promiscuity of this network, and specifically, that NRG can regulate Tyr phosphorylation of the EGFR, if erbB-3 or erbB-4 are present, and that NRG-responsiveness of erbB-3 can be conferred by co-expression of any of the EGFR, neu, or erbB-4. Thus NRG promotes extensive interactions of erbB-3 and erbB-4 with the other receptors. Similarly, EGF and β-cellulin promote extensive cross-talk. The interactions do show some specificity, however, and additional specificity may be masked by variable receptor levels. The greatest specificity was seen with the erbB-3/erbB-4 combination: NRG stimulated Tyr phosphorylation of both receptors, but β-cellulin stimulated only

erbB-4. Thus there are qualitative as well as quantitative specificities in transmodulating interactions.

C.Coupling to IL-3 independent growth. Although the specific responses of Ba/F3 cells will not necessarily predict those in other backgrounds, they reveal the extent to which combinatorial receptor interactions govern these responses. With a single exception, activation of either neu or the EGFR by any of the peptides was associated with cell survival. In contrast, erbB-3 had no apparent influence beyond enabling NRG to activate EGFR and neu. erbB-4 failed to promote survival or proliferation on its own or in concert with erbB-3. Il-3-dependent proliferation was only seen with activation of certain receptor combinations: EGFR + neu, and EGFR + erbB-4.

D.Summary. Each factor tested activates a unique group of single receptors, and a unique set of combinations. Receptor response is determined first by binding specificity, second by kinase activity (erbB-3 requires a functional partner), third by the availability of transmodulation partners, which shows some degree of specificity.The strong response to EGFR + erbB-4 suggests either that each of two signals provided, for example by the EGFR and by erbB-4, is necessary for proliferation, or that the heterodimeric combination has unique coupling abilities. *The exact complement of erbB family receptors dictates the cellular response to each factor.*

II.NRG Intracellular Domain

The cytoplasmic domain of NRG family EGF-related growth factors is unusual in showing extraordinary diversity of regulation by splicing, and in that some forms have unusually long cytoplasmic tails of unknown function (over 400 amino acids). We hypothesize that these tails are likely to themselves transmit signals so that binding of NRG ecto-domains to the cognate receptors results in bidirectional signaling. As a first step in addressing this problem, we have begun

to clone and express these factors in transient and stable transfected cell lines. This work was complicated by some expression problems that were ultimately solved by changing expression vectors. We have now succeeded in expressing full length and cytoplasmic deletion forms of NRG both transiently and stably in NIH 3T3 cells. We are using both myc- and flag- epitope tagged molecules to enable recovery of the proteins by immunoprecipitation and immunoblotting. Once the biological activity of these molecules in activating receptors has been established, the next step will be to use the assays described in the original proposal to identify signaling functions associated with the cytoplasmic domain. Initial studies will focus on activation of intracellular signaling pathways, and association with catenins. In parallel we have begun to make and characterize molecular clones for use in 2-hybrid screening of interacting proteins.

This work marks progress on Task3 b, which is on schedule. 3c is on hold for now because we are using epitope tags, and 3a has so far yielded negative results.

III.Biological Activities of NRG and TGF-α.

We have re-arranged our original schedule by moving the mammary tissue pellet implant experiments up to years 1 and 2. Pellets were impregnated with NRG or TGF-α without or with estrogen/progesterone. Implants were inserted into the the surgically-exposed number 4 mammary fat pad of 32 da Balb/c females, with growth factor-free pellets used as controls in the contralateral mammary fat pad. Four days after implantation whole mounts were prepared and stained with hematoxylin.We favor this approach because the epithelium responds in the context of normal matrix and hormonal environment and hence is highly physiological, and has the collateral advantages of being fast, manipulable and inexpensive. Although there is considerable literature for both EGF and TGF-α, the NRGs have not been investigated in this format. These experiments are still

in progress, but initial results show that NRG-α is potent in induction of both ductal extension, branching and in addition promotes some lobuloalveolar development. This is significant because it shows for the first time the in vivo activity of this hormone, and because it suggests that NRG can indeed act as a differentiation factor as has been hypothesized. Further work is underway to compare the responses to TGF-α and to NRG-β.

This work marks partial completion of Task 4b in the Statement of work, one year ahead of schedule.

CONCLUSIONS

The most significant conclusions can be drawn from the work summarized in Table 1:

We have analyzed the NRG-dependent responses conferred upon the Ba/F3 mouse pro-B-lymphocyte cell line by expression of the four erbB family receptors, singly and in pairwise combinations. In the presence of appropriate co-receptors, NRG regulated the tyrosine phosphorylation of all four erbB family receptors. While some of the NRG-induced interactions between erbB family receptors observed had been predicted from previous work, we demonstrate here for the first time that in the presence of erbB-3 or erbB-4, NRG regulates tyrosine phosphorylation of the EGFR, and that the presence of the EGFR, neu, or erbB-4 enables NRG to regulate tyrosine phosphorylation of erbB3. Furthermore, NRG induces IL-3-independent survival or proliferation in only a subset of the lines that exhibit NRG-induced receptor tyrosine phosphorylation. Thus the biological responses to NRG are specified at several different levels of regulation.

Previous analyses of NRG-induced signaling by erbB family receptors have been carried out with a few receptor combinations in a variety of cell backgrounds. In mammary cells, NRGs induce neu tyrosine phosphorylation, can be cross-linked to neu, and binding is increased by neu overexpression, at first suggesting that neu is a receptor for NRG. However, NRG fails to induce neu tyrosine phosphorylation and/or bind neu when neu is expressed in fibroblasts, ovarian cells, CHO cells , T-lymphoid cells, or COS-7 cells. Moreover, NRG binds erbB-3 or erbB-4 , and co-expression of erbB-3 or erbB-4 with neu confers NRG responsiveness upon neu. This has led to the general working hypothesis that activation of neu by NRG requires the presence of erbB-3 or erbB-4. The present data are compatible with this conclusion, and extend the model to include NRG regulation of the EGFR. The EGFR and erbB-3 alone fail to respond

to NRG for two different reasons. The EGFR does not bind NRG, whereas erbB-3 binds, but is impaired for kinase activity. The stimulation of tyrosine phosphorylation of neu by NRG might suggest direct activation of neu by NRG, but in view of previously published work, is more likely to reflect interaction with endogenous erbB-3. However, erbB-4 is able to bind and respond to NRG directly.

NRG induces extensive cross-talk among receptors expressed in binary combinations (Table 1). Either erbB-3 or erbB-4, both of which bind NRG, enable regulation of the EGFR by NRG. This is the first evidence that NRG can regulate EGFR signaling. As predicted from earlier work, NRG stimulates tyrosine phosphorylation of both receptors in the neu + erbB3, neu + erbB-4, and erbB-3 + erbB-4 cell lines. Coexpression of EGFR, neu, or erbB-4 with erbB-3 permits NRG induction of erbB-3 tyrosine phosphorylation. Although earlier work showed that expression of neu enhances tyrosine phosphorylation and NRG regulation of erbB-3 [Carraway, *et al.*, 1994], that work was done in COS-7 cells, which express significant basal amounts of neu and EGFR. Thus the present work demonstrates for the first time that *de novo* expression of either the EGFR, neu, or of erbB-4 enables hormone-regulated phosphorylation of erbB-3. Endogenous erbB receptor expression in Ba/F3 cells played a limited, yet significant role in specifying responses to NRG stimulation in these experiments. While endogenous erbB-3 expression permits NRG stimulation of exogenous neu tyrosine phosphorylation, NRG does not stimulate receptor tyrosine phosphorylation in cells that express exogenous EGFR only. Perhaps the level of endogenous erbB-3 expression in Ba/F3 cells is insufficient to permit NRG-induced EGFR tyrosine phosphorylation. Alternatively, intrinsic differences between the exogenous human and endogenous mouse proteins may result in

the differing capacities to undergo NRG-induced heterotypic receptor interactions.

While NRG can stimulate the tyrosine phosphorylation of each receptor under the appropriate conditions, the diversity of biological responses to NRG indicates that there must be additional mechanisms by which biological responses to NRG are specified. The patterns of NRG-induced stimulation of erbB receptor tyrosine phosphorylation and IL-3 independent survival or proliferation demonstrates that there are several hierarchical levels at which biological responses to NRG are apparently specified.

First, responsiveness to NRG requires the expression of erbB-3 or erbB-4. Previous work and results presented here establish that NRG can not bind or stimulate tyrosine phosphorylation of erbB family receptors in the absence of erbB-3 or erbB-4 expression. Biological responses to NRG are also specified by the intrinsic kinase activity of the erbB family receptor(s) stimulated by NRG, since the kinase-deficient erbB-3 requires the presence of a co-receptor for hormone-regulated phosphorylation.

Distinct biological responses to NRG are also conferred by ligand-induced coupling of different erbB family receptors to different signaling pathways. NRG enables the IL-3 independent survival of cell lines expressing neu, probably through NRG-induced activation of neu via erbB-3. However, NRG does not enable the IL-3 independent survival of EGFR + erbB-3 cells, or of erbB-4 cells, even though NRG stimulates receptor tyrosine phosphorylation in these lines. This demonstrates that neu has signaling properties distinct from those of the EGFR, erbB-3, or erbB-4, and is consistent with earlier work showing that different erbB family receptors can activate different signaling pathways and responses. Neither NRG nor EGF induces IL-3-independent proliferation of cells that individually express erbB-4 or EGFR (Table 1). Yet, NRG stimulates IL-3-

independent proliferation in the EGFR + erbB-4 cell line. One simple explanation would be that IL-3-independent proliferation requires activation of two independent pathways, one of which is activated by the EGFR, and one by erbB-4. An interesting alternative would be that EGFR and erbB-4 phosphorylation sites differ in ligand-induced EGFR/erbB-4 heterodimers than in ligand-induced receptor homodimers owing to substrate specificity of the receptor catalytic domains and steric considerations in the cross-phosphorylation reaction. This would permit recruitment of unique signaling proteins to the heterodimer, resulting in unique biological responses. Thus, the diversity of hormone-regulated outputs from this receptor outwork may extend beyond the simple combinatorial possibilities.

Since EGF and NRG bind to different receptors, it can be predicted that individual members of the EGF family of ligands activate different constellations of erbB family receptors, so that these different ligands will yield distinct patterns of biological responses. Evidence presented here supports this prediction. Previous reports demonstrated that betacellulin binds to the A431 human adenocarcinoma cell line and the MDA-MB-453 human breast carcinoma cell line, both of which overexpress the EGFR. This binding was quenched by the addition of an excess of EGF, suggesting that betacellulin is a ligand for the EGFR. However, the effect of betacellulin on EGFR tyrosine phosphorylation and signaling and the possibility that betacellulin might activate other erbB family receptors were not assessed. We have shown that in Ba/F3 cells expressing only a single ectopic erbB family receptor, betacellulin stimulates the tyrosine phosphorylation of both the EGFR and, surprisingly, erbB-4 (Table 1). This is consistent with the observation that radiolabeled betacellulin binds specifically to EGFR and erbB-4, but not to neu (Plowman, *et al.*, in preparation). Control experiments performed in parallel demonstrated that radiolabeled amphiregulin

and EGF bound only to EGFR and radiolabeled NRG-β bound only to erbB-4, as previously reported. Thus, betacellulin exhibits activities that are distinct from those displayed by EGF, which activates the EGFR alone, and NRG-β, which activates erbB-3 and erbB-4 (Table 1). Furthermore, in this first comprehensive analysis of erbB family transmodulation for both betacellulin and EGF, we find that EGF can transmodulate erbB-4 in the EGFR + erbB-4 cell line (Table 1). We also demonstrate that betacellulin stimulates a pattern of receptor transmodulation that is qualitatively distinct from the patterns stimulated by EGF and NRG-β.

With one exception, betacellulin, EGF, and NRG-β transmodulated the tyrosine phosphorylation of all four erbB family receptors in cell lines that express any receptor for each ligand (Table 1). Thus, differences in ligand activities in the double recombinant cell lines can be predicted by differences in activities in the single recombinant cell lines. For example, NRG-β activates erbB-3, while betacellulin does not activate neu or erbB-3. Not surprisingly, in cells expressing neu + erbB-3, NRG-β stimulates the phosphorylation of both receptors, while betacellulin does not stimulate the phosphorylation of either receptor in this cell line (Table 1). The single exception is the response of the erbB-3 + erbB-4 (3+4) cell line to betacellulin. Both betacellulin and NRG-β stimulate erbB-4 tyrosine phosphorylation in the single recombinant cell line. However, in 3+4 cells, NRG-β stimulates the tyrosine phosphorylation of both receptors, while betacellulin stimulates the tyrosine phosphorylation of erbB-4 but not of erbB-3 (Table 1). Nonetheless, because NRG-β binds erbB-3, it is not clear that this absence of erbB-3 tyrosine phosphorylation is due to differences between betacellulin- or NRG-β-induced erbB-3 transmodulation.

Previous work demonstrated that different erbB family receptors or combinations of receptors couple to distinct cellular signaling pathways. For

example, EGFR activation stimulates the tyrosine phosphorylation of four proteins that are not highly phosphorylated following neu activation [63]. Furthermore, activated erbB-3 stimulated higher levels of phosphatidylinositol 3-kinase than EGFR did [64, 65, 66], and it has been suggested that EGFR and neu bind the adapter protein GRB2, but erbB-3 does not (Prigent and Gullick, 1994). These different coupling capacities of the erbB family receptors can be correlated to specific biological responses. In Ba/F3 cells, activation of neu stimulates IL-3 independent survival, while activation of EGFR and erbB-4 together stimulates IL-3 independent proliferation .

We found that betacellulin stimulates IL-3 independent survival or proliferation in neu + erbB-4 cells and in every cell line that expresses EGFR. In contrast, EGF stimulated IL-3 independence only in those cell lines that express EGFR while NRG-β stimulated IL-3 independence only in the EGFR + erbB-4 cell line and in those cell lines that express neu (Table 1). Therefore, with a single exception, the minimal requirement for IL-3 independence is activation of either EGFR or neu. The exception is that betacellulin and EGF, but not NRG-β, stimulated IL-3 independent survival in the EGFR + erbB-3 cell line (Table 1). This lack of response to NRG-β may merely reflect the lower level of EGFR phosphorylation stimulated by NRG-β in this cell line. On the other hand, the absence of biological response may reflect the different mechanism by which the EGFR is activated. We demonstrated that coupling of these multiple receptor species to cellular signaling pathways acts in a non-additive manner in specifying biological responses (Table 1). Accordingly, while activation of EGFR or neu by themselves stimulated IL-3 independent survival, activation of EGFR and either neu or erbB-4 together stimulated IL-3 independent proliferation (Table 1).

Our data suggest that differences in NRG-β, EGF, and betacellulin activities play a significant role in specifying the proliferation and differentiation of human tissues *in vivo*. Increases in the expression and/or signaling of erbB family receptors plays a significant role in tumors of mammary or neuroectodermal origin [4]. Because betacellulin, NRG-β, and EGF have distinct biological activities that apparently reflect their differing abilities to activate receptor signaling, it may be possible to develop antagonists that specifically disrupt signaling by a single EGF family ligand and may inhibit the genesis or growth of malignancies without disrupting the activity of other EGF family ligands in the same tissue.

Future work. We plan to continue with the original game plan in the Statement of Work. Our efforts so far have been highly successful, and there is no compelling reason to change strategies in the near future.

REFERENCES

1. Bargmann, C. I., M. C. Hung, and R. A. Weinberg. 1986. Nature 310:226-230.
2. Stern, D. F., P. A. Heffernan, and R. A. Weinberg. 1986. Mol. Cell. Biol. 6:1729-1740.
3. Coussens, L., T. L. Yang-Feng, Y.-C. Liao, E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, A. Levinson, and A. Ullrich. 1985. Science 230:1132-1139.
4. Hynes, N. E., and D. F. Stern. 1994. Biochemica et Biophysica Acta Reviews on Cancer 1198:165-184.
5. King, C. R., S. M. Swain, L. Porter, S. M. Steinberg, M. E. Lippman, and E. P. Gelmann. 1989. Cancer Res. 49:4185-4191.
6. Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire. 1987. Science 235:177-182.
7. Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, A. Ullrich, and M. F. Press. 1989. Science 244:707-712.
8. Ali, I. U., G. Campbell, R. Lidereau, and R. Callahan. 1988. Science 240:1795-1796.
9. Anbazhagan, R., R. D. Gelber, R. Bettelheim, A. Goldhirsch, and B. A. Gusterson. 1991. Ann. Oncol. 2:47-53.
10. Gasparini, G., W. J. Gullick, P. Bevilacqua, R. C. Sainsbury, S. Meli, P. Boracchi, A. Testolin, G. La Malfa, and F. Pozza. 1992. J. Clin. Oncol. 10:686-695.
11. Hynes, N. E. 1993. Sem. in Cancer Biol. 4:19-26.
12. Kallioniemi, O.-P., A. Kallioniemi, W. Kurisu, A. Thor, L.-C. Chen, H. S. Smith, F. M. Waldman, D. Pinkel, and J. W. Gray. 1992. Proc. Natl. Acad. Sci. USA 89:5321-5325.

13. Levine, M. N., and I. Andrulis. 1992. J. Clin. Oncol. 10:1034-1036.

14. van de Vijver, M. J., J. L. Peterse, W. J. Mooi, P. Wisman, J. Lomans, O. Dalesio, and R. Nusse. 1988. N. Engl. J. Med. 319:1239-1245.

15. Clark, G. M., and W. L. McGuire. 1991. Cancer Res. 51:944-948.

16. Allred, D. C., G. M. Clark, R. Molina, A. K. Tandon, S. J. Schnitt, K. W. Gilchrist, C. K. Osborne, D. C. Tormey, and W. L. McGuire. 1992. Hum. Pathol. 23:974-979.

17. Kraus, M. H., N. C. Popescu, S. C. Amsbaugh, and C. R. King. 1987. EMBO 6:605-610.

18. Siegel, P. M., D. L. Dankort, W. R. HArdy, and W. J. Muller. 1994. Molecular and Cellular Biology 14:7068-7077.

19. Paterson, M. C., K. D. Dietrich, J. Danyluk, A. H. G. Paterson, A. W. Lees, N. Jamil, J. Hanson, H. Jenkins, B. E. Krause, W. A. McBlain, D. J. Slamon, and R. M. Fourney. 1991. Cancer Res. 51:556-567.

20. Paik, S., R. Hazan, E. R. Fisher, R. E. Sass, B. Fisher, C. Redmond, J. Schlessinger, M. E. Lippman, and C. R. King. 1990. J. Clin. Oncol. 8:103-112.

21. Hudziak, R. M., J. Schlessinger, and A. Ullrich. 1987. Proc. Natl. Acad. Sci. USA 84:7159-7163.

22. Di Fiore, P. P., J. H. Pierce, M. H. Kraus, O. Segatto, C. R. King, and S. A. Aaronson. 1987. Science 237:178-182.

23. Muller, W. J., E. Sinn, P. K. Pattengale, R. Wallace, and P. Leder. 1988. Cell 54:105-115.

24. Bouchard, L., L. Lamarre, P. J. Tremblay, and P. Jolicoeur. 1989. Cell 57:931-936.

25. Guy, C. T., M. A. Webster, M. Schaller, T. J. Parsons, R. D. Cardiff, and W. J. Muller. 1992. Proceedings of the National Academy of Sciences USA 89:10578-10582.

26. Baselga, J., and J. Mendelsohn. 1994. Pharmac. Ther. 64:127-154.

27. Toyoda, H., T. Komurasaki, D. Uchida, Y. Takayama, T. Isobe, T. Okuyama, and K. Hanada. 1995. Journal of Biological Chemistry 270:7495-7500.

28. Plowman, G. D., J. M. Green, V. L. McDonald, M. G. Neubauer, C. M. Disteche, G. J. Todaro, and M. Shoyab. 1990. Mol. Cell. Biol. 10:1969-1981.

29. Shing, Y., G. Christofori, D. Hanahan, Y. Ono, R. Sasada, K. Igarashi, and J. Folkman. 1993. Science 259:1604-1607.

30. Derynck, R. 1992. Adv. Cancer Res. 58:27-52.

31. Higashiyama, S., J. A. Abraham, J. Miller, J. C. Fiddes, and M. Klagsbrun. 1991. Science 251:936-939.

32. Stern, D. F., and M. P. Kamps. 1988. EMBO Journal 7:995-1001.

33. Connelly, P. A., and D. F. Stern. 1990. Proc. Natl. Acad. Sci. USA 87:6054-6057.

34. King, C. R., I. Borrello, F. Bellot, P. Comoglio, and J. Schlessinger. 1988. EMBO 7:1647-1651.

35. Johnson, G., B. Kannan, M. Shoyab, and K. Stromberg. 1993. J. Biol;. Chem. 268:2294-2931.

36. Spivak-Kroizman, T., D. Rotin, D. Pinchasi, A. Ullrich, J. Schlessinger, and I. Lax. 1992. J. Biol. Chem. 267:8056-8063.

37. Goldman, R., R. Ben Levy, E. Peles, and Y. Yarden. 1990. Biochem. 29:11024-11028.

38. Wada, T., X. Qian, and M. I. Greene. 1990. Cell 61:1339-1347.

39. Peles, E., R. Ben-Levy, E. Tzahar, N. Liu, D. Wen, and Y. Yarden. 1993. EMBO Journal 12:961-971.

40. Kokai, Y., J. N. Meyers, T. Wada, V. I. Brown, C. M. LeVea, J. G. Davis, K. Dobashi, and M. I. Greene. 1989. Cell 58:287-292.

41. Graus-Porta, D., R. R. Beerli, and N. E. Hynes. 1995. Molecular and Cellular Biology 15:1182-1191.

42. Di Fiore, P. P., O. Segatto, W. G. Taylor, S. A. Aaronson, and J. H. Pierce. 1990. Science 248:79-83.

43. Earp, J. S., T. L. Dawson, X. Li, and H. Yu. 1995. Breast Cancer Research and Treatment 35:115-132.

44. Lemke, G. 1993. Nature 362:291-292.

45. Holmes, W. E., M. X. Sliwkowski, R. W. Akita, W. J. Henzel, J. Lee, J. W. Park, D. Yansura, N. Abadi, H. Raab, G. D. Lewis, H. M. Shepard, W.-J. Kuang, W. I. Wood, D. V. Goeddel, and R. L. Vandlen. 1992. Science 256:1205-1210.

46. Lupu, R., R. Colomer, G. Zugmaier, J. Sarup, M. Shepard, D. Slamon, and M. E. Lippman. 1990. Science 249:1552-1555.

47. Lupu, R., R. Colomer, B. Kannan, and M. E. Lippman. 1992. Proc. Natl. Acad. Sci. USA 89:2287-2291.

48. Peles, E., S. S. Bacus, R. A. Koski, H. S. Lu, D. Wen, S. G. Ogden, R. Ben Levy, and Y. Yarden. 1992. Cell 69:205-216.

49. Wen, D., E. Peles, R. Cupples, S. V. Suggs, S. S. Bacus, Y. Luo, G. Trail, S. Hu, S. M. Silbiger, R. B. Levy, R. A. Koski, H. S. Lu, and Y. Yarden. 1992. Cell 69:559-572.

50. Marchionni, M. A., A. D. J. Goodearl, M. S. Chen, O. Berminghan-McDonogh, C. Kirk, M. Hendricks, F. Danehy, D. Misumi, J. Sudhalter, K. Kobayashi, D. Wroblewski, C. Lynch, M. Baldassare, I. Hiles, J. B. Davis, J. J. Hsuan, N. F. Totty, M. Otsu, R. N. McBurney, M. D. Waterfield, P. Stroobant, and D. Gwynne. 1993. Nature 362:312-318.

51. Corfas, G., D. L. Falls, and G. D. Fischbach. 1993. Proc. Natl. Acad. Sci. USA 90:1624-1628.

52. Plowman, G., J. Green, J.-M. Culouscou, G. Carlton, V. Rothwell, and S. Buckley. 1993. Nature 366:473-475.

53. Sliwkowski, M., G. Schaefer, R. W. Akita, J. A. Lofgren, V. D. Fitzpatrick, A. Nuijens, B. M. Fendly, R. A. Cerione, R. L. Vandlen, and K. L. Carraway III. 1994. Journal of Biological Chemistry 269:14661-14665.

54. Guy, P. M., J. V. Platko, L. C. Cantley, R. A. Cerione, and I. K L Carraway. 1994. Proceedings of the National Academy of Sciences USA 91:8132-8136.

55. Huang, S. S., and J. S. Huang. 1992. J. Biol. Chem. 267:11508-11512.

56. Dobashi, K., J. G. Davis, Y. Mikami, J. K. Freeman, J. Hamuro, and M. I. Greene. 1991. Proc. Natl. Acad. Sci. USA 88:8582-8586.

57. Normanno, N., C.-F. Qi, W. J. Gullick, G. Persico, Y. Yarden, D. Wen, G. Plowman, N. Kenney, G. Johnson, N. Kim, R. Brandt, I. Martinez-Lacaci, R. B. Dickson, and D. S. Salomon. 1993. Int. J. Oncol. 2:903-911.

58. Liscia, D. S., G. Merlo, F. Ciardiello, N. Kim, G. H. Smith, R. Callahan, and D. S. Salomon. 1990. Developmental Biology 140:123-131.

59. LeJeune, S., R. Leek, E. Horak, G. Plowman, M. Greenall, and A. L. Harris. 1993. Cancer Research 53:3597-3602.

60. Culouscou, J.-M., G. D. Plowman, G. W. Carlton, J. M. Green, and M. Shoyab. 1993. J. Biol. Chem. 268:18407-18410.

61. Riese II, D. J., Y. Bermingham, T. M. van Raaij, S. Buckley, G. D. Plowman, and D. F. Stern. Oncogene. *in press*

62. Riese II, D. J., T. M. van Raaij, G. D. Plowman, G. C. Andrews, and D. F. Stern. 1995. Mol.Cell.Biol. 15:5770-5776.

63. Fazioli, F., D. P. Bottaro, L. Minichiello, A. Auricchio, W. T. Wong, O. Segatto, and P. P. DiFiore. 1992. J. Biol. Chem. 267:5155-5161.

64. Fedi, P., J. H. Pierce, P. P. Di Fiore, and M. H. Kraus. 1994. Mol. Cell. Biol. 14:492-500.

65. Soltoff, S. P., and I. Carraway Kermit L. 1994. Molecular and Cellular Biology 14:3550-3558.

66. Carraway, K. L., III, S. P. Soltoff, A. J. Diamonti, and L. C. Cantley. 1995. J. Biol. Chem. 270:7111-7116.

MOLECULAR AND CELLULAR BIOLOGY, Oct. 1995, p. 5770–5776 Vol. 15, No. 10
0270-7306/95/$04.00+0

The Cellular Response to Neuregulins Is Governed by Complex Interactions of the erbB Receptor Family

DAVID J. RIESE II,[1] TOM M. VAN RAAIJ,[1] GREGORY D. PLOWMAN,[2]† GLENN C. ANDREWS,[3] AND DAVID F. STERN[1]*

Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520-8023[1]; Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121[2]; and Pfizer Central Research, Groton, Connecticut 06340[3]

Received 11 May 1995/Returned for modification 15 June 1995/Accepted 17 July 1995

Deregulated signaling by the four members of the epidermal growth factor receptor tyrosine kinase family (erbB family) is implicated in the genesis or progression of human cancers. However, efforts to analyze signaling by these receptors have been hampered by the diversity of ligands and extensive interreceptor cross talk. We have expressed the four human erbB family receptors, singly and in pairwise combinations, in a pro-B-lymphocyte cell line (Ba/F3) and investigated the range of interactions activated by the epidermal growth factor homology domain of the agonist neuregulin β. The results provide the first comprehensive analysis of the response of this receptor family to a single peptide agonist. This peptide induced complex patterns of receptor tyrosine phosphorylation and regulation of Ba/F3 cell survival and proliferation. These data demonstrate the existence of several previously undocumented receptor interactions driven by neuregulin.

Deregulated signaling by the four receptor tyrosine kinases encoded by the *erbB* gene family (erbB-1/epidermal growth factor receptor [EGFR], neu/erbB-2/HER2, erbB-3/HER3, and erbB-4/HER4) is implicated in human mammary cancer, ovarian cancer, gastric cancer, and glioblastoma (reviewed in reference 19). Understanding the normal and pathological functions of these receptors requires that their regulation by hormones be elucidated. One complication is that there are at least 15 different agonists for erbB family receptors, including EGF, transforming growth factor α, amphiregulin, betacellulin, heparin-binding EGF-like growth factor, and the several differentially spliced variants of the neuregulins (NRGs), also known as gp30 (27), heregulins (18), neu differentiation factors (35, 54), glial growth factors (28), and acetylcholine receptor-inducing activity (5, 12). Some of these factors bind to and activate signaling by more than one receptor. Moreover, these ligands stimulate nonadditive receptor interactions in cells expressing multiple erbB receptor family members. For example, EGF activates neu when coexpressed with the EGFR, but EGF does not bind or activate neu expressed on its own (22, 50). This transmodulation activation of neu by EGFR apparently works through the formation of EGF-driven receptor heterodimers (15, 53).

NRGs were initially identified as candidate neu ligands by their ability to induce neu tyrosine phosphorylation. The longest forms of NRG contain several different modular domains, including a kringle fold, a C-2 immunoglobulin-like domain, a putative heparan sulfate proteoglycan attachment site, sites for N- and O-linked glycosylation, an EGF homology domain, a hydrophobic membrane-spanning domain, and an intracellular domain of variable length (6, 18, 28, 35, 54). Tissue-specific alternative splicing of NRG transcripts from a single gene results in many NRG isoforms containing different sets of these motifs. Moreover, alternative splicing also produces two types of EGF domain, designated α and β (55). α and β isoforms have different biological activities, which may in part reflect their differential binding affinities to cells expressing receptors for NRG (26).

NRGs are likely to play a significant role in regulating cellular proliferation and differentiation in vivo. NRGs were initially purified from medium conditioned by *ras*-transformed Rat-1 fibroblasts (35) or by the MDA-MB-231 human mammary tumor cell line (18), suggesting that NRGs establish or maintain the growth-transformed phenotype. NRG also affects the proliferation and differentiation of cultured mammary cells. NRG stimulates (18) or inhibits (35, 54) the in vitro proliferation of human mammary tumor cells, which frequently overexpress erbB family receptors (reviewed in reference 19), while NRG stimulates proliferation and milk protein synthesis in a cultured mouse mammary epithelial cell line (29). NRG may also promote wound healing. A single NRG isoform accelerates epidermal migration via increased terminal differentiation of epidermal cells and stimulates integrin expression in the epidermis during wound healing, while wounding stimulates NRG expression in dermal fibroblasts adjacent to the wound (9). NRG also modulates the differentiation and proliferation of neuroectodermal cells. NRGs act as glial cell growth factors (28), may specify a glial cell fate for neural crest stem cells (45), appear to mediate axon-induced mitogenesis of Schwann cells (32), and stimulate acetylcholine receptor synthesis at neuromuscular junctions (5, 12, 20). Furthermore, NRG expression patterns suggest important functions in neurogenesis and in mesenchymal-epithelial cell interactions during development (6, 28, 30, 33).

The physiological responses to agonists for erbB family receptors depend on their ability to coordinately activate multiple receptors that are differentially expressed and have different signaling capabilities. Although NRGs were initially purified by their ability to induce neu tyrosine phosphorylation and were thought to be ligands for neu, NRG does not bind neu and/or induce neu tyrosine phosphorylation in a variety of cell types or in solution (7, 36, 39, 48, 51). Instead, NRG binds erbB-3 (2, 23, 48, 51) and erbB-4 (7, 38, 39, 51). Coexpression of erbB-3 or erbB-4 with neu permits NRG-induced tyrosine

* Corresponding author. Mailing address: Department of Pathology, Yale University School of Medicine, P.O. Box 208023, New Haven, CT 06520-8023. Phone: (203) 785-4832. Fax: (203) 785-7467. Electronic mail address: STERN@BIOMED.MED.YALE.EDU.

† Present address: Sugen, Inc., Redwood City, CA 94063-4720.

phosphorylation of neu, presumably through the formation of neu–erbB-3 or neu–erbB-4 heterodimers (2, 23, 39, 48). Despite the many combinatorial possibilities afforded by assortment of four different receptors, interactions of erbB family members with their agonists have been investigated only in a piecemeal fashion. Receptors of different species origins have been mixed in gene transfer experiments, only a subset of receptor combinations have been tested, and interpretation is hampered by the variety of cell backgrounds used and in many cases by the failure to determine the endogenous erbB family receptor expression in the cell lines used. Finally, the hormone-regulated coupling of different erbB family receptors and combinations of receptors to different downstream signaling pathways has not been systematically investigated for any EGF family agonist, including NRGs.

To address these issues, we have undertaken a parallel analysis of the aggregate signaling potential of this receptor family by expressing all four human erbB family receptors, singly and in each pairwise combination, in a uniform cell background. We have used the resulting cell lines for the first comprehensive evaluation of NRG-induced erbB family receptor activation and coupling to cellular responses. The results reveal the pattern of activation of these receptors by NRG and identify novel ligand-induced interactions among these receptors. Moreover, these data suggest several distinct mechanisms by which biological responses are specified by interactions among erbB family receptors and their agonists.

MATERIALS AND METHODS

Cell lines and cell culture. The Ba/F3 mouse pro-B-lymphocyte cell line (34) and its derivatives were grown in RPMI (Gibco/BRL) supplemented with 10% fetal calf serum (Sigma) and interleukin-3 (IL-3) supplied as 10% conditioned medium from the WEHI-3B mouse myelomonocytic leukemia cell line (8). Ba/F3 derivatives transformed with constructs expressing erbB family receptors were grown in medium supplemented with 200 μg of G418 (Gibco/BRL) per ml.

Plasmid constructions. The *Sac*II-*Xho*I fragment of pCO12EGFR (52), which contains the full-length human EGFR cDNA, was subcloned into the *Sma*I site of pBluescript SK−, generating pSKEGFR. The EGFR expression vector pLXSN-EGFR used in the experiments described here was constructed by cloning the 4.2-kb *Xho*I fragment of pSKEGFR, which contains the complete human EGFR cDNA, into the *Xho*I site of the recombinant retroviral expression vector plasmid pLXSN, which carries a neomycin resistance gene under the transcriptional control of the simian virus 40 early promoter (31). The neu expression vector pLXSN-Long-Neu was constructed by cloning the 4.8-kb *Nru*I-to-*Dra*I fragment of pCDNEU (39), which contains the complete human neu cDNA as well as 714 bp of vector sequences 5′ to the neu transcriptional start site, into the *Hpa*I site of pLXSN. Subsequently, the vector sequences 5′ to the neu transcriptional start site were removed by cloning a 4.1-kb *Xho*I fragment of pLXSN-Long-Neu into the *Xho*I site of pLXSN, generating the neu expression vector pLXSN-Neu used in these studies. The erbB-3 expression vector pLXSN-erbB-3 was constructed by cloning the 4.3-kb *Bss*HII fragment of pBSHER3X (40), which contains the complete human erbB-3 cDNA, into the *Hpa*I site of pLXSN. The erbB-4 expression vector pLXSN-erbB-4 was constructed by cloning the 4.6-kb *Sna*BI-to-*Sma*I fragment of pCH4M2 (38), which contains the complete human erbB-4 cDNA, into the *Hpa*I site of pLXSN.

Generation of recombinant Ba/F3 derivatives. Ten micrograms of a single expression vector directing the expression of an erbB family receptor or 5 μg of each of a pair of expression vectors was linearized by digestion with restriction endonucleases and ligated to form concatemers. These were electroporated into 2×10^7 Ba/F3 cells in 0.5 ml of Tris-buffered saline, using a 0.4-cm gap cuvette and a Bio-Rad Gene Pulser set at 200 V and 960 μF. Cells were immediately diluted into 50 ml of culture medium, incubated for 48 h at 37°C, and then seeded in 96-well dishes at 5×10^4 cells per well in medium supplemented with 400 μg of G418 per ml. Drug-resistant lines were expanded and screened for expression of the appropriate erbB family receptor(s). Positive lines were subcloned by limiting dilution and rescreened for receptor expression to ensure homogeneity. The cell lines characterized here are named as follows: LXSN/1 (vector control); EGFR/3; neu/5 and neu/12C; erbB-3/3; erbB-4/7; EGFR + neu/5D; EGFR + erbB-3/4A; EGFR + erbB-4/2A; neu + erbB-3/7A; neu + erbB-4/15A; and erbB-3 + erbB-4/2B.

The ranked order of receptor expression in the double recombinant cell lines is as follows. For EGFR expression, EGFR + erbB-4/2A is higher than EGFR + neu/5D, which is higher than EGFR + erbB-3/4A. For neu expression, neu + erbB-4/15A is marginally higher than EGFR + neu/5D, which is markedly higher than neu + erbB-3/7A. The levels of ErbB-3 expression are similar in the EGFR + erbB-3/4A, neu + erbB-3/7A, and erbB-3 + erbB-4/2B cell lines, while the levels of erbB-4 expression are similar in the EGFR + erbB-4/2A, neu + erbB-4/15A, and erbB-3 + erbB-4/2B cell lines.

Stimulation and analysis of erbB family tyrosine phosphorylation. A total of 2×10^8 recombinant Ba/F3 cells were washed in phosphate-buffered saline (PBS) and resuspended in 50 ml of RPMI supplemented with IL-3. The cells were incubated for 6 h at 37°C, washed in PBS, and resuspended in 1 to 2 ml of PBS. Remaining steps were performed cold or on ice. The cells were transferred in two or three 0.5- to 1.0-ml portions to microcentrifuge tubes. A chemically synthesized NRG β 65-mer peptide (1) corresponding to amino acids 177 to 241 of the NRG β1 isoform (amino acid residues are numbered according to reference 18) or the anti-neu agonistic monoclonal antibody (MAb) TAb 250 (24, 46) was added at a final concentration of 94 ng/ml (NRG) or 10 μg/ml (TAb 250). Control samples remained untreated or were treated with NRG dilution buffer. Following a 10-min incubation, cells were pelleted and incubated for 10 min in 1 ml of EBC lysis buffer (37), which is a Tris-buffered 120 mM sodium chloride solution containing 0.5% Nonidet P-40. Debris was pelleted by centrifugation, and the supernatants were transferred to a fresh tube and diluted 1:3 in EBC to facilitate sample handling. The protein content in each sample was assayed by using Coomassie blue assay reagent (Pierce), and a volume of lysate containing 2 mg of protein was used for each immunoprecipitation.

EGFR was immunoprecipitated with 900 ng of anti-EGFR MAb 528 (14) and 7.2 μg of rabbit anti-mouse antibody 31188 (Pierce); neu was immunoprecipitated with 2 μg of anti-neu MAb TAb 250 (24) and 12 μg of rabbit anti-mouse antibody or with 1 μg of anti-neu MAb FSP-16 (17) and 5 μg of rabbit anti-mouse antibody; erbB-3 was immunoprecipitated with 200 ng of anti-erbB-3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology); erbB-4 was immunoprecipitated with 1 μg of anti-erbB-4 rabbit polyclonal antiserum SC-283 (Santa Cruz Biotechnology). Specificity of antireceptor antibodies was verified by testing each precipitating antibody for cross-reactivity with cell lines expressing heterologous receptors. All immunoprecipitation mixtures were incubated at 4°C for 2 h, after which the immune complexes were collected by incubation at 4°C with 50 μl of a 10% (vol/vol) suspension of fixed and washed *Staphylococcus aureus* (IGSL-10; The Enzyme Center). Immune complexes were washed three times with NET-N (37) and were eluted from *S. aureus* by boiling in 150 μl of protein sample buffer (37). Samples were divided equally, electrophoresed on separate 7.5% acrylamide–0.17% bisacrylamide–0.1% SDS gels (44), and transferred to nitrocellulose (11) for immunoblotting with either the antiphosphotyrosine MAb 4G10 (Upstate Biotechnology, Inc.) or antibodies specific for receptors. Antibody binding was detected with horseradish peroxidase-coupled sheep anti-mouse antibody NA931 (Amersham) or horseradish peroxidase-coupled donkey anti-rabbit antibody NA934 (Amersham) and enhanced chemiluminescence reagent RPN2106 (Amersham). Immunoblotting antibodies were sheep anti-EGFR polyclonal antibody 06-129 (Upstate Biotechnology Inc.), rabbit anti-sheep antibody 31240 (Pierce), rabbit anti-neu antibody Ab1 (PC04; Oncogene Science), mouse anti-erbB-3 MAb 2F12 (21), and rabbit anti-erbB-4 polyclonal antibody SC-283 (Santa Cruz Biotechnology).

RESULTS

The mouse Ba/F3 pro-B-lymphocyte cell line was chosen for expression of erbB family members because the survival and proliferation of the cells are tightly regulated by exogenous growth factor (IL-3) and because erbB family receptors have not been found to be expressed in mammalian hematopoietic cells. Immunoprecipitation and immunoblotting experiments did not reveal endogenous expression of any erbB family receptors in these cells (42). Nonetheless, we further assessed endogenous receptor expression by PCR amplification of reverse-transcribed transcripts (RT-PCR assay), the most sensitive assay available. RT-PCR analysis of erbB family receptor transcription by using probes homologous to murine erbB family receptor genes in a control Ba/F3 cell line or in Ba/F3 cell lines expressing exogenous human EGFR, neu, erbB-3, or erbB-4 demonstrated that these lines lacked endogenous murine EGFR, neu, or erbB-4 transcription (data not shown). Surprisingly, however, all of the Ba/F3 cell lines tested exhibited detectable levels of endogenous erbB-3 transcription (data not shown). This novel finding implies that erbB family receptors and their ligands may play important roles in the differentiation, expansion, or growth transformation of cells of a B-lymphocyte lineage.

cDNAs directing the expression of erbB family receptors were introduced into Ba/F3 cells to generate clonal lines that

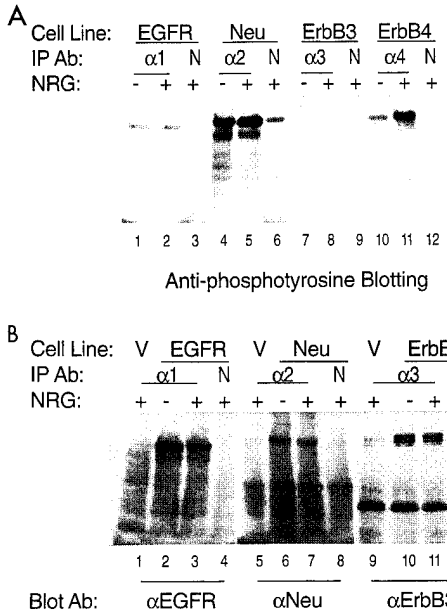


FIG. 1. Regulation of receptor tyrosine phosphorylation by NRG in single recombinant Ba/F3 derivatives. Untreated or NRG-stimulated cell lines were immunoprecipitated with antireceptor antibodies, and portions of immunoprecipitates were analyzed by immunoblotting with antiphosphotyrosine (A) or antireceptor (B) antibodies. V refers to LXSN (vector only) cells. The neu-expressing line used is neu/5. Immunoprecipitating antibodies (IP Ab): α1, anti-EGFR; α2, anti-neu; α3, anti-erbB-3; α4, anti-erbB-4; N, normal mouse or rabbit serum. +, immunoprecipitations of lysates from NRG-treated cells; −, immunoprecipitations of lysates from untreated cells.

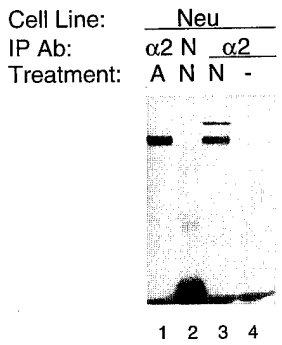


FIG. 2. Regulation of neu/12C cells by NRG. neu/12C cells were incubated with NRG dilution buffer (−), the agonistic anti-neu antibody TAb 250 (46) (A), or NRG (N). The immunoprecipitating antibody (IP Ab) used for immunoprecipitating lysates was anti-neu antibody (α2) or normal mouse serum (N); after immunoprecipitation, lysates were analyzed by immunoblotting with antiphosphotyrosine. The band above neu (lane 3) was not observed in other trials.

express the four different human receptors, singly and in combination. The resulting panel of cell lines was stimulated with a chemically synthesized NRG 65-mer peptide (amino acids 177 to 241 of NRG β1), which encompasses the EGF homology domain and is sufficient for induction of receptor tyrosine phosphorylation (1, 18). Regulation of tyrosine phosphorylation of each receptor by NRG was evaluated by immunoprecipitating the receptors and immunoblotting with antiphosphotyrosine (Fig. 1A and 3A) and antireceptor (Fig. 1B and 3B) antibodies.

Among cell lines expressing a single exogenous receptor (Fig. 1A), NRG failed to stimulate tyrosine phosphorylation of the EGFR (lanes 1 and 2) or erbB-3 (lanes 7 and 8). In contrast, NRG strongly activated tyrosine phosphorylation of erbB-4 (lanes 10 and 11). Since high basal tyrosine phosphorylation of neu in the neu/5 cell line may have obscured the effect of NRG (lanes 4 and 5), we isolated an independent Ba/F3 derivative, designated neu/12C, that expresses considerably less neu than the neu/5 cell line. In this cell line, NRG clearly activated neu tyrosine phosphorylation (Fig. 2, lanes 3 and 4).

In most of the double recombinant cell lines, NRG unambiguously stimulated tyrosine phosphorylation of both erbB family receptors (Fig. 3A; summarized in Table 1). Since the four erbB family receptors have distinct electrophoretic mobilities in most combinations, coprecipitation of heterologous dimerization partners would have been detected. However, coprecipitation was not observed under these conditions. Significantly, the results for the double recombinant cell lines are not simply additive with the responses of single cell lines. For example, NRG does not stimulate tyrosine phosphorylation of the uniquely expressed EGFR, but exogenous coexpression of erbB-3 or erbB-4 with EGFR enabled NRG to regulate EGFR tyrosine phosphorylation. Similarly, while NRG did not stimulate tyrosine phosphorylation of erbB-3 alone, coexpression of EGFR, neu, or erbB-4 permitted activation of erbB-3. Thus, NRG can regulate the tyrosine phosphorylation of each erbB family receptor provided that the appropriate coreceptor is expressed.

While NRG can stimulate the tyrosine phosphorylation of all four erbB family receptors, activation of different receptors or combinations of receptors may specify unique biological responses through coupling of each receptor to distinct cellular signaling pathways. We investigated this possibility by determining whether NRG stimulation enabled survival or growth of the various Ba/F3 derivatives independent of IL-3. Ectopic expression of a number of receptors in Ba/F3 cells permits receptor regulation by the cognate ligands, which in some cases relieves dependence on IL-3 for survival or growth: expression of the erythropoietin receptor with Friend spleen focus-forming virus gp55 permits IL-3-independent proliferation (25). Similarly, EGF stimulation of Ba/F3 cells expressing exogenous EGFR results in EGFR tyrosine phosphorylation and increased cellular DNA synthesis (4, 47), while stimulation of Ba/F3 derivatives expressing exogenous platelet-derived growth factor receptor with platelet-derived growth factor results in receptor tyrosine phosphorylation and IL-3-independent proliferation (43).

In the absence of NRG, all of the Ba/F3 derivatives, even those lines that display substantial basal receptor tyrosine phosphorylation, remained dependent on IL-3 for survival (Table 2). Likewise, NRG stimulation does not confer IL-3-independent survival or growth on control Ba/F3 cells. In cell lines expressing a single exogenous receptor, expression of neu, but not EGFR, erbB-3, or erbB-4, permitted NRG-dependent survival of Ba/F3 cells (Table 2). Indeed, all cell lines engineered to express neu (neu/5, neu/12C [42], EGFR + neu, neu + erbB-3, and neu + erbB-4) survived in the presence of NRG. This survival appears to be dependent on the amount of neu expression, as the neu/5 cell line, which expresses more neu than the other cell lines, also exhibited the strongest IL-3-independent response to NRG, while the neu/12C and neu + erbB-3 cell lines, which express less neu than the other cell lines, exhibited the weakest response to NRG treatment. NRG failed to promote the IL-3-independent survival or proliferation of erbB-4, EGFR + erbB-3, and erbB-3 + erbB-4 lines, even though NRG regulated receptor phosphorylation in these

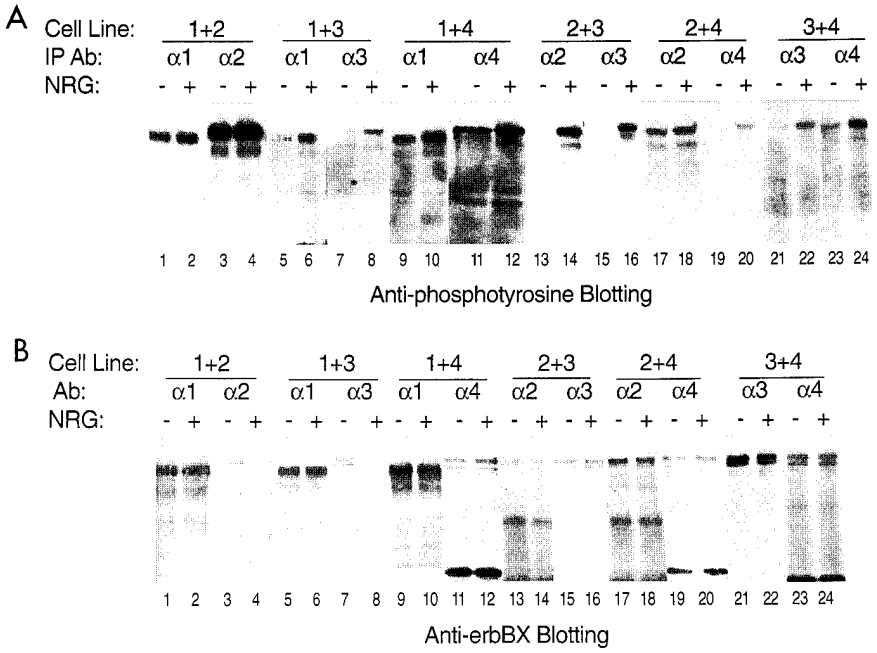


FIG. 3. Regulation of receptor tyrosine phosphorylation in double recombinant Ba/F3 derivatives. Untreated or NRG-treated cells were immunoprecipitated with antireceptor antibodies and analyzed by immunoblotting with antiphosphotyrosine (A) or antireceptor (B) antibodies. Cell lines: 1+2, EGFR + neu; 1+3, EGFR + erbB-3; 1+4, EGFR + erbB-4; 2+3, neu + erbB-3; 2+4, neu + erbB-4; 3+4, erbB-3 + erbB-4. The immunoprecipitating (IP) and/or immunoblotting antibodies (Ab): α1, anti-EGFR antibody; α2, anti-neu antibody; α3, anti-erbB-3 antibody; α4, anti-erbB-4 antibody. +, immunoprecipitations of lysates from NRG-treated cells; −, immunoprecipitations of lysates from untreated cells.

lines (Table 1). Furthermore, while NRG had no effect on the survival of cells expressing either the EGFR or erbB-4 alone, stimulation of EGFR + erbB-4 cells enabled this line to reach saturation densities comparable to those induced by IL-3 (Table 2). For this cell line, NRG acts not as an IL-3-independent survival factor but as a proliferative agent. Hence, not only is NRG-regulated receptor phosphorylation not sufficient for coupling to a cellular response, but the quality of the response is governed by the exact combination of regulated receptors present.

TABLE 1. Summary of NRG-induced erbB family receptor tyrosine phosphorylation and IL-3-independent growth or survival

Cell line	Receptor	NRG-stimulated tyrosine phosphorylation[a]	IL-3-independent growth in the presence of NRG[b]
LXSN		−	N
EGFR		−	S
neu/12C		+	S
erbB-3		−	N
erbB-4		+	N
EGFR + neu	EGFR	+/−	S
	neu	+/−	
EGFR + erbB-3	EGFR	+	N
	erbB-3	+	
EGFR + erbB-4	EGFR	+	P
	erbB-4	+	
neu + erbB-3	neu	+	S
	erbB-3	+	
neu + erbB-4	neu	+/−	S
	erbB-4	+	
erbB-3 + erbB-4	erbB-3	+	N
	erbB-4	+	

[a] Results are abstracted from Fig. 1 to 3 and similar, unpublished data. +, increased receptor tyrosine phosphorylation over basal levels; −, no increase in receptor tyrosine phosphorylation; +/−, borderline results due to high basal levels of receptor tyrosine phosphorylation.

[b] Adapted from Table 2 and similar, unpublished data. N, NRG does not enhance cell survival; S, NRG sustains viable cells; P, NRG induces IL-3-independent proliferation.

DISCUSSION

We have analyzed the NRG-dependent responses conferred upon the Ba/F3 mouse pro-B-lymphocyte cell line by expres-

TABLE 2. Ba/F3 density in response to IL-3 starvation and NRG stimulation[a]

Cell line	Viable cell saturation density (10^3 cells/ml)		
	IL-3 free	IL-3	NRG
LXSN	2	1,765	4
EGFR	1	1,301	3
neu/5	1	2,030	310
erbB-3	<1	1,800	<1
erbB-4	<1	1,583	8
EGFR + neu	2	1,104	162
EGFR + erbB-3	1	1,393	<1
EGFR + erbB-4	3	1,851	1,093
neu + erbB-3	<1	1,258	50
neu + erbB-4	1	1,664	291
erbB-3 + erbB-4	<1	1,475	3

[a] For each trial and treatment, Ba/F3 cells made quiescent by growth to saturation density were plated at a density of 100×10^3/ml in two independently seeded flasks containing medium lacking IL-3 (IL-3 free), medium supplemented with IL-3 (IL-3), or medium lacking IL-3 but supplemented with NRG β 65-mer at a final concentration of 9.4 ng/ml (NRG). Over the next 4 days, cells were stained with trypan blue and counted in a hemacytometer to determine the density of viable cells. For all treatments and cell lines, cells reached viable cell saturation densities with approximately the same kinetics. Data shown are values averaged from two or three independent trials.

sion of the four erbB family receptors, singly and in pairwise combinations. In the presence of appropriate coreceptors, NRG regulated the tyrosine phosphorylation of all four erbB family receptors. While some of the NRG-induced interactions between erbB family receptors observed had been predicted from previous work, we demonstrate here for the first time that in the presence of erbB-3 or erbB-4, NRG regulates tyrosine phosphorylation of the EGFR and that the presence of the EGFR, neu, or erbB-4 enables NRG to regulate tyrosine phosphorylation of erbB3. Furthermore, NRG induces IL-3-independent survival or proliferation in only a subset of the lines that exhibit NRG-induced receptor tyrosine phosphorylation. Thus, the biological responses to NRG are specified at several different levels of regulation.

Previous analyses of NRG-induced signaling by erbB family receptors have been carried out with a few receptor combinations in a variety of cell backgrounds. In mammary cells, NRGs induce neu tyrosine phosphorylation and can be cross-linked to neu, and binding is increased by neu overexpression (36), at first suggesting that neu is a receptor for NRG. However, NRG fails to induce neu tyrosine phosphorylation and/or bind neu when neu is expressed in fibroblasts, ovarian cells (36), CHO cells (7, 39), T-lymphoid cells (39), or COS-7 cells (48), and NRG does not bind to solubilized neu extracellular domains (51). Moreover, NRG binds erbB-3 (2, 23, 48, 51) or erbB-4 (7, 38, 39, 51), and coexpression of erbB-3 or erbB-4 with neu confers NRG responsiveness upon neu, probably through the formation of neu/erbB-3 or neu/erbB-4 heterodimers (2, 23, 39, 48). This finding has led to the general working hypothesis that activation of neu by NRG requires the presence of erbB-3 or erbB-4.

The data presented in this report are compatible with this conclusion and extend the model to include NRG regulation of the EGFR. The EGFR and erbB-3 alone fail to respond to NRG for two different reasons. The EGFR does not bind NRG (18), whereas erbB-3 binds but is impaired for kinase activity (16). The stimulation of tyrosine phosphorylation of neu by NRG might suggest direct activation of neu by NRG but, in view of previously published work, is more likely to reflect interaction with endogenous erbB-3. However, erbB-4 is able to bind and respond to NRG directly.

NRG induces extensive cross talk among receptors expressed in binary combinations (Table 1). Either erbB-3 or erbB-4, both of which bind NRG, enables regulation of the EGFR by NRG. This is the first evidence that NRG can regulate EGFR signaling. As predicted from earlier work, NRG stimulates tyrosine phosphorylation of both receptors in the neu + erbB3, neu + erbB-4, and erbB-3 + erbB-4 cell lines. Coexpression of EGFR, neu, or erbB-4 with erbB-3 permits NRG induction of erbB-3 tyrosine phosphorylation. Although earlier work showed that expression of neu enhances tyrosine phosphorylation and NRG regulation of erbB-3 (2), that work was done in COS-7 cells, which express significant basal amounts of neu and EGFR. Thus, the present work demonstrates for the first time that de novo expression of either the EGFR, neu, or erbB-4 enables hormone-regulated phosphorylation of erbB-3. Endogenous erbB receptor expression in Ba/F3 cells played a limited yet significant role in specifying responses to NRG stimulation in these experiments. While endogenous erbB-3 expression permits NRG stimulation of exogenous neu tyrosine phosphorylation, NRG does not stimulate receptor tyrosine phosphorylation in cells that express exogenous EGFR only. Perhaps the level of endogenous erbB-3 expression in Ba/F3 cells is insufficient to permit NRG-induced EGFR tyrosine phosphorylation. Alternatively, intrinsic differences between the exogenous human and endogenous mouse proteins may result in the different capacities to undergo NRG-induced heterotypic receptor interactions.

While NRG can stimulate the tyrosine phosphorylation of each receptor under the appropriate conditions, the diversity of biological responses to NRG indicates that there must be additional mechanisms by which biological responses to NRG are specified. The patterns of NRG-induced stimulation of erbB receptor tyrosine phosphorylation and IL-3-independent growth and proliferation demonstrate that there are several hierarchical levels at which biological responses to NRG are apparently specified.

First, responsiveness to NRG requires the expression of erbB-3 or erbB-4. Previous work and results presented here establish that NRG cannot bind or stimulate tyrosine phosphorylation of erbB family receptors in the absence of erbB-3 or erbB-4 expression. Biological responses to NRG are also specified by the intrinsic kinase activity of the erbB family receptor(s) stimulated by NRG, since the kinase-deficient erbB-3 requires the presence of a coreceptor for hormone-regulated phosphorylation.

Distinct biological responses to NRG are also conferred by ligand-induced coupling of different erbB family receptors to different signaling pathways. NRG enables the IL-3-independent survival of cell lines expressing neu, probably through NRG-induced activation of neu via erbB-3. However, NRG does not enable the IL-3-independent survival of EGFR + erbB-3 cells or of erbB-4 cells, even though NRG stimulates receptor tyrosine phosphorylation in these lines. This finding demonstrates that neu has signaling properties distinct from those of the EGFR, erbB-3, or erbB-4, consistent with earlier work showing that different erbB family receptors can activate different signaling pathways and responses (3, 10, 13, 21, 41, 49). Neither NRG nor EGF induces IL-3-independent proliferation of cells that individually express erbB-4 or EGFR (Table 2 and reference 42), yet NRG stimulates IL-3-independent proliferation in the EGFR + erbB-4 cell line. One simple explanation would be that IL-3-independent proliferation requires activation of two independent pathways, one activated by the EGFR and one activated by erbB-4. An interesting alternative would be that EGFR and erbB-4 phosphorylation sites are different in ligand-induced EGFR/erbB-4 heterodimers than in ligand-induced receptor homodimers owing to substrate specificity of the receptor catalytic domains and steric considerations in the cross-phosphorylation reaction. This would permit recruitment of unique signaling proteins to the heterodimer, resulting in unique biological responses. Thus, the diversity of hormone-regulated outputs from this receptor outwork may extend beyond the simple combinatorial possibilities.

Since EGF and NRG bind to different receptors, it can be predicted that individual members of the EGF family of ligands activate different constellations of erbB family receptors, so that these different ligands will yield distinct patterns of biological responses. Preliminary evidence supports this prediction. Three different members of the EGF family induce different patterns of erbB family receptor tyrosine phosphorylation and IL-3-independent growth in the Ba/F3 derivatives described here (42). Given the multitude of roles that erbB family receptors and their ligands apparently play in diverse biological processes such as neurogenesis, neuromuscular signaling, tumorigenesis, wound healing, and the regulation of mesenchymal-epithelial cell interactions, it is likely that all of the mechanisms described here play a significant part in specifying responses to ligand stimulation in vivo.

ACKNOWLEDGMENTS

We thank Brad Guarino and James D. Moyer, Pfizer Central Research, for providing the synthetic NRG β. We thank Mitchell Goldfarb for advice on electroporating and selecting Ba/F3 cells. We thank Daniel DiMaio and Alan D'Andrea for the generous gifts of Ba/F3 and WeHi cells, and we thank Daniela Drummond-Barbosa and other members of the DiMaio laboratory for advice on handling Ba/F3 cells. We thank John Koland and Hideo Masui for the generous gifts of anti-erbB antibodies and Duanzhi Wen for the gifts of recombinant NRGs. We also thank Susan Hwang, Michael DiGiovanna, Debbie Hayden, and other members of the Stern laboratory for their advice, reagents, and technical support.

This work was supported by Public Health Service grant CA-45708-06 from the National Cancer Institute and by grant DAMD-17-94-J-4476 from the U.S. Army Medical Research and Materiel Command to D.F.S. D.J.R. was supported by Public Health Service postdoctoral training grant HD-07149 from the National Cancer Institute and by postdoctoral fellowship DAMD-17-94-J-4036 from the U.S. Army Medical Research and Materiel Command. T.M.V.R. was supported by a Dutch Cancer Society fellowship.

REFERENCES

1. **Barbacci, E. G., B. C. Guarino, J. G. Stroh, D. H. Singleton, K. J. Rosnack, J. D. Moyer, and G. C. Andrews.** 1995. The structural basis for the specificity of EGF and heregulin binding. J. Biol. Chem. **270:**9585–9589.
2. **Carraway, K. L., III, M. X. Sliwkowski, R. Akita, J. V. Platko, P. M. Guy, A. Nuijens, A. J. Diamonti, R. L. Vandlen, L. C. Cantley, and R. A. Cerione.** 1994. The *erbB3* gene product is a receptor for heregulin. J. Biol. Chem. **269:**14303–14306.
3. **Carraway, K. L., III, S. P. Soltoff, A. J. Diamonti, and L. C. Cantley.** 1995. Heregulin stimulates mitogenesis and phosphatidylinositol 3-kinase in mouse fibroblasts transfected with *erbB2/neu* and *erbB3*. J. Biol. Chem. **270:**7111–7116.
4. **Collins, M. K. L., J. Downward, A. Miyajima, K. Maruyama, K.-I. Arai, and R. C. Mulligan.** 1988. Transfer of functional EGF receptors to an IL3-dependent cell line. J. Cell. Physiol. **137:**293–298.
5. **Corfas, G., D. L. Falls, and G. D. Fischbach.** 1993. ARIA, a protein that stimulates acetylcholine receptor synthesis, also induces tyrosine phosphorylation of a 185-kDa muscle transmembrane protein. Proc. Natl. Acad. Sci. USA **90:**1624–1628.
6. **Corfas, G., K. M. Rosen, H. Aratake, R. Krauss, and G. D. Fischbach.** 1995. Differential expression of ARIA isoforms in the rat brain. Neuron **14:**103–115.
7. **Culouscou, J.-M., G. D. Plowman, G. W. Carlton, J. M. Green, and M. Shoyab.** 1993. Characterization of a breast cancer cell differentiation factor that specifically activates the HER4/p180^{erbB4} receptor. J. Biol. Chem. **268:**18407–18410.
8. **Daley, G. Q., and D. Baltimore.** 1988. Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210$^{bcr/abl}$ protein. Proc. Natl. Acad. Sci. USA **85:**9312–9316.
9. **Danilenko, D. M., B. D. Ring, J. Z. Lu, J. E. Tarpley, D. Chang, M. Liu, D. Wen, and G. F. Pierce.** 1995. Neu differentiation factor upregulates epidermal migration and integrin expression in excisional wounds. J. Clin. Invest. **95:**842–851.
10. **DiFiore, P. P., O. Segatto, W. G. Taylor, S. A. Aaronson, and J. H. Pierce.** 1990. EGF receptor and *erb*B-2 tyrosine kinase domains confer cell specificity for mitogenic signaling. Science **248:**79–83.
11. **DiGiovanna, M. P., and D. F. Stern.** 1995. Activation state specific monoclonal antibody detects tyrosine phosphorylated p185$^{neu/erbB-2}$ in a subset of human breast tumors overexpressing this receptor. Cancer Res. **55:**1946–1955.
12. **Falls, D. L., K. M. Rosen, G. Corfas, W. S. Lane, and G. D. Fischbach.** 1993. ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell **72:**801–815.
13. **Fedi, P., J. H. Pierce, P. P. DiFiore, and M. H. Kraus.** 1994. Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase Cγ or GTPase-activating protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. Mol. Cell. Biol. **14:**492–500.
14. **Gill, G. N., T. Kawamoto, C. Cochet, A. Le, J. D. Sato, H. Masui, C. McLeod, and J. Mendelsohn.** 1984. Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. J. Biol. Chem. **259:**7755–7760.
15. **Goldman, R., R. Ben Levy, E. Peles, and Y. Yarden.** 1990. Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. Biochemistry **29:**11024–11028.
16. **Guy, P. M., J. V. Platko, L. C. Cantley, R. A. Cerione, and K. L. Carraway III.** 1994. Insect cell-expressed p180^{erbB3} possesses an impaired tyrosine kinase activity. Proc. Natl. Acad. Sci. USA **91:**8132–8136.
17. **Harwerth, I. M., W. Wels, B. M. Marte, and N. E. Hynes.** 1992. Monoclonal antibodies against the extracellular domain of the erbB-2 receptor function as partial ligand agonists. J. Biol. Chem. **267:**15160–15167.
18. **Holmes, W. E., M. X. Sliwkowski, R. W. Akita, W. J. Henzel, L. Lee, J. W. Park, D. Yansura, N. Abadi, H. Raab, G. D. Lewis, H. M. Shepard, W.-J. Kuang, W. I. Wood, D. V. Goeddel, and R. L. Vandlen.** 1992. Identification of heregulin, a specific activator of p185^{erbB2}. Science **256:**1205–1210.
19. **Hynes, N. E., and D. F. Stern.** 1994. The biology of *erbB-2/neu/HER-2* and its role in cancer. Biochim. Biophys. Acta **1198:**165–184.
20. **Jo, S. A., X. Zhu, M. A. Marchionni, and S. J. Burden.** 1995. Neuregulins are concentrated at nerve-muscle synapses and activate ACh-receptor gene expression. Nature (London) **373:**158–161.
21. **Kim, H.-H., S. L. Sierke, and J. G. Koland.** 1994. Epidermal growth factor-dependent association of phosphatidylinositol 3-kinase with the *erb*B3 gene product. J. Biol. Chem. **269:**24747–24755.
22. **King, C. R., I. Borrello, F. Bellot, P. Comoglio, and J. Schlessinger.** 1988. EGF binding to its receptor triggers a rapid tyrosine phosphorylation of the *erb*B-2 protein in the mammary tumor cell line SK-BR-3. EMBO J. **7:**1647–1651.
23. **Kita, Y. A., J. Barff, Y. Luo, D. Wen, D. Brankow, S. Hu, N. Liu, S. A. Prigent, W. J. Gullick, and M. Nicolson.** 1994. NDF/heregulin stimulates the phosphorylation of Her3/erbB3. FEBS Lett. **349:**139–143.
24. **Langton, B. C., M. C. Crenshaw, L. A. Chao, S. G. Stuart, R. W. Akita, and J. E. Jackson.** 1991. An antigen immunologically related to the external domain of gp185 is shed from nude mouse tumors overexpressing the c-*erb*B-2 (HER-2/*neu*) oncogene. Cancer Res. **51:**2593–2598.
25. **Li, J.-P., A. D. D'Andrea, H. F. Lodish, and D. Baltimore.** 1990. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature (London) **343:**762–764.
26. **Lu, H. S., D. Chang, J. S. Philo, K. Zhang, L. O. Narhi, N. Liu, M. Zhang, J. Sun, J. Wen, D. Yanagihara, D. Karunagaran, Y. Yarden, and B. Ratzkin.** 1995. Studies on the structure and function of glycosylated and nonglycosylated *neu* differentiation factors. J. Biol. Chem. **270:**4784–4791.
27. **Lupu, R., R. Colomer, G. Zugmaier, J. Sarup, M. Shepard, D. Slamon, and M. E. Lippman.** 1990. Direct interaction of a ligand for the *erb*B2 oncogene product with the EGF receptor and p185^{erbB2}. Science **249:**1552–1555.
28. **Marchionni, M. A., A. D. J. Goodearl, M. S. Chen, O. Bermingham-McDonogh, C. Kirk, M. Hendricks, F. Danehy, D. Misumi, J. Sudhalter, K. Kobayashi, D. Wroblewski, C. Lynch, M. Baldassare, I. Hiles, J. B. Davis, J. J. Hsuan, N. F. Totty, M. Otsu, R. N. McBurney, M. D. Waterfield, P. Stroobant, and D. Gwynne.** 1993. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature (London) **362:**312–318.
29. **Marte, B. M., M. Jeschke, D. Graus-Porta, D. Taverna, P. Hofer, B. Groner, Y. Yarden, and N. E. Hynes.** 1995. Neu differentiation factor/heregulin modulates growth and differentiation of HC11 mammary epithelial cells. Mol. Endocrinol. **9:**14–23.
30. **Meyer, D., and C. Birchmeier.** 1994. Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development. Proc. Natl. Acad. Sci. USA **91:**1064–1068.
31. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques **7:**980–990.
32. **Morrissey, T. K., A. D. O. Levi, A. Nuijens, M. X. Sliwkowski, and R. P. Bunge.** 1995. Axon-induced mitogenesis of human Schwann cells involves heregulin and p185erbB2. Proc. Natl. Acad. Sci. USA **92:**1431–1435.
33. **Orr-Urtreger, A., L. Trakhtenbrot, R. Ben-Levy, D. Wen, G. Rechavi, P. Lonai, and Y. Yarden.** 1993. Neuronal expression and chromosomal mapping of Neu differentiation factor to 8p12-p21. Proc. Natl. Acad. Sci. USA **90:**1867–1871.
34. **Palacios, R., and M. Steinmetz.** 1985. IL-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes *in vivo*. Cell **41:**727–734.
35. **Peles, E., S. S. Bacus, R. A. Koski, H. S. Lu, D. Wen, S. G. Ogden, R. Ben Levy, and Y. Yarden.** 1992. Isolation of the Neu/HER-2 stimulatory ligand: a 44kd glycoprotein that induces differentiation of mammary tumor cells. Cell **69:**205–216.
36. **Peles, E., R. Ben-Levy, E. Tzahar, N. Lu, D. Wen, and Y. Yarden.** 1993. Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships. EMBO J. **12:**961–971.
37. **Petti, L., L. A. Nilson, and D. DiMaio.** 1991. Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. EMBO J. **10:**845–855.
38. **Plowman, G. D., J.-M. Culouscou, G. S. Whitney, J. M. Green, G. W. Carlton, L. Foy, M. G. Neubauer, and M. Shoyab.** 1993. Ligand-specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. Proc. Natl. Acad. Sci. USA **90:**1746–1750.
39. **Plowman, G. D., J. M. Green, J.-M. Culouscou, G. W. Carlton, V. M. Rothwell, and S. Buckley.** 1993. Heregulin induces tyrosine phosphorylation of HER4/p180^{erbB4}. Nature (London) **366:**473–475.
40. **Plowman, G. D., G. S. Whitney, M. G. Neubauer, J. M. Green, V. L. McDonald, G. J. Todaro, and M. Shoyab.** 1990. Molecular cloning and expres-

sion of an additional epidermal growth factor receptor-related gene. Proc. Natl. Acad. Sci. USA **87:**4905–4909.
41. **Prigent, S. A., and W. J. Gullick.** 1994. Identification of c-erbB-3 binding sites for phosphatidylinositol 3′-kinase and SHC using an EGF receptor/c-erbB-3 chimera. EMBO J. **13:**2831–2841.
42. **Riese, D. J., II, T. M. van Raaij, Y. Bermingham, G. D. Plowman, and D. F. Stern.** Unpublished results.
43. **Satoh, T., W. J. Fantl, J. A. Escobedo, L. T. Williams, and Y. Kaziro.** 1993. Platelet-derived growth factor receptor mediates activation of *ras* through different signaling pathways in different cell types. Mol. Cell. Biol. **13:**3706–3713.
44. **Sefton, B., K. Beemon, and T. Hunter.** 1979. Comparison of the expression of the *src* gene of Rous sarcoma virus in vitro and in vivo. J. Virol. **28:**957–971.
45. **Shah, N. M., M. A. Marchionni, I. Isaacs, P. Stroobant, and D. J. Anderson.** 1994. Glial growth factor restricts mammalian neural crest stem cells to a glial fate. Cell **77:**349–360.
46. **Shawver, L. K., E. Mann, S. S. Elliger, T. C. Dugger, and C. L. Arteaga.** 1994. Ligand-like effects induced by anti-c-erbB-2 antibodies do not correlate with and are not required for growth inhibition of human carcinoma cells. Cancer Res. **54:**1367–1373.
47. **Shibuya, H., M. Yoneyama, J. Ninomiya-Tsuji, K. Matsumoto, and T. Taniguchi.** 1992. IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for c-myc. Cell **70:**57–67.
48. **Sliwkowski, M. X., G. Schaefer, R. W. Akita, J. A. Lofgren, V. D. Fitzpatrick, A. Nuijens, B. M. Fendly, R. A. Cerione, R. L. Vandlen, and K. L. Carraway III.** 1994. Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin. J. Biol. Chem. **269:**14661–14665.
49. **Soltoff, S. P., K. L. Carraway III, S. A. Prigent, W. J. Gullick, and L. C. Cantley.** 1994. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol. Cell. Biol. **14:**3550–3558.
50. **Stern, D. F., and M. P. Kamps.** 1988. EGF-stimulated tyrosine phosphorylation of p185^{neu}: a potential model for receptor interactions. EMBO J. **7:**995–1001.
51. **Tzahar, E., G. Levkowitz, D. Karunagaran, L. Yi, E. Peles, S. Lavi, D. Chang, J. Liu, A. Yayon, D. Wen, and Y. Yarden.** 1994. ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor/heregulin isoforms. J. Biol. Chem. **269:**25226–25233.
52. **Velu, T. J., L. Beguinot, W. C. Vass, M. C. Willingham, G. T. Merlino, I. Pastan, and D. R. Lowy.** 1987. Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene. Science **238:**1408–1410.
53. **Wada, T., X. Qian, and M. I. Greene.** 1990. Intermolecular association of the p185^{neu} protein and EGF receptor molecules modulates EGF receptor function. Cell **61:**1339–1347.
54. **Wen, D., E. Peles, R. Cupples, S. V. Suggs, S. S. Bacus, Y. Luo, G. Trail, S. Hu, S. M. Silbiger, R. Ben Levy, R. A. Koski, H. S. Lu, and Y. Yarden.** 1992. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell **69:**559–572.
55. **Wen, D., S. V. Suggs, D. Karunagaran, N. Liu, R. L. Cupples, Y. Luo, A. M. Janssen, N. Ben-Baruch, D. B. Trollinger, V. L. Jacobsen, S.-Y. Meng, H. S. Lu, S. Hu, D. Chang, W. Yang, D. Yanigahara, R. A. Koski, and Y. Yarden.** 1994. Structural and functional aspects of the multiplicity of Neu differentiation factors. Mol. Cell. Biol. **14:**1909–1919.

Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-β

David J. Riese II[1], Yamilée Bermingham[1], Tom M. van Raaij[1], Sharon Buckley[2], Gregory D. Plowman[2], and David F. Stern[1]*

[1]Department of Pathology
Yale University School of Medicine,
New Haven, CT 06520-8023

[2]Sugen, Inc.
515 Galveston Drive
Redwood City, CA 94063-4720

Running Title: Betacellulin and erbB receptor signaling

Keywords: Betacellulin, EGF, Neuregulin, EGF Receptor, ErbB receptors, Signaling

* Corresponding Author:
Department of Pathology
Yale University School of Medicine
P.O. Box 208023
New Haven, CT 06520-8023
Telephone: (203) 785-4832
FAX: (203) 785-7467

Abstract

Betacellulin is a member of the epidermal growth factor (EGF) family. These soluble proteins are ligands for one or more of the four receptor tyrosine kinases encoded by the erbB gene family (erbB-1/epidermal growth factor receptor [EGFR], neu/erbB-2/HER2 , erbB-3/HER3 , and erbB-4/HER4). While evidence suggests that betacellulin is a ligand for the EGFR, the ability of betacellulin to regulate other erbB family receptors has not been analyzed. Previously we engineered derivatives of the mouse Ba/F3 hematopoietic cell line to ectopically express erbB family receptors, singly and in pairwise combinations. We have stimulated this panel of cell lines with betacellulin and two other EGF family members, EGF itself and neuregulin-β (NRG-β). In the cell lines expressing a single erbB family receptor, betacellulin not only stimulated EGFR tyrosine phosphorylation, but it activated erbB-4 as well. Furthermore, in the double recombinant Ba/F3 derivatives, betacellulin stimulated a complex pattern of receptor phosphorylation distinct from the patterns activated by NRG-β and EGF. Moreover, betacellulin stimulated a complex pattern of interleukin-3 independence in the Ba/F3 derivatives distinct from those activated by NRG-β and EGF. These data identify a novel receptor for betacellulin and establish that different EGF family ligands activate distinct patterns of receptor phosphorylation and coupling to cellular signaling pathways.

Introduction

The epidermal growth factor (EGF) family consists of at least 15 members, including epidermal growth factor, transforming growth factor alpha (TGFα), amphiregulin (AR), betacellulin, heparin-binding-EGF-like growth factor (HB-EGF), cripto, epiregulin, and the several differentially-spliced variants of neuregulin (NRGs), also known as heregulins, neu differentiation factors, gp30, glial growth factors, and acetylcholine receptor inducing activity [Reviewed in Groenen, *et al.*, 1994]. These factors activate a family of four receptor tyrosine kinases encoded by the erbB gene family (erbB-1/epidermal growth factor receptor [EGFR], neu/erbB-2/HER2 , erbB-3/HER3 , and erbB-4/HER4). The patterns of receptor activation stimulated by EGF family ligands is very complex; some of these peptides bind to and activate signaling by more than one erbB family receptor. Furthermore, in cell lines expressing multiple erbB receptor family members, these ligands can activate erbB family receptors that do not bind these ligands when the receptors are expressed individually, a process called transmodulation [Reviewed in Hynes and Stern, 1994; Earp, *et al.*, 1995]. For example, EGF activates neu when coexpressed with the EGFR, but EGF does not bind or activate neu expressed on its own [Akiyama, *et al.*, 1988; King, *et al.*, 1988; Stern and Kamps, 1988; Connelly and Stern, 1990]. It has been proposed that transmodulation occurs through the formation of EGF-driven receptor heterodimers and receptor cross-phosphorylation [Goldman, *et al.*, 1990; Wada, *et al.*, 1990; Qian, *et al.*, 1992; Spivak-Kroizman, *et al.*, 1992]. Not only does EGF induce erbB receptor transmodulation, but every EGF family member tested thus far has this activity [Stern and Kamps, 1988; Johnson, *et al.*, 1993; Plowman, *et al.*, 1993; Carraway, *et al.*, 1994; Kita, *et al.*, 1994; Sliwkowski, *et al.*, 1994; Karunagaran, *et al.*, 1995; Riese, *et al.*, in press].

Betacellulin was initially identified as a factor in the conditioned medium of a mouse pancreatic β cell carcinoma (insulinoma) cell line that is mitogenic for

Balb/C 3T3 cells. The 80 amino acid mature betacellulin protein is derived from a 177 amino acid membrane-bound precursor and contains the six conserved cysteine residues that are arranged in a characteristic pattern common to all of the EGF family members. Furthermore, betacellulin has significant overall homology to mature EGF, TGF-α, AR, HB-EGF, and NRGs [Sasada, *et al.*, 1993; Shing, *et al.*, 1993; Reviewed in Groenen, *et al.*, 1994]. Binding of human recombinant betacellulin to the A431 human adenocarcinoma cell line, which overexpresses the EGFR, can be competed with an excess of EGF, suggesting that betacellulin is a ligand for the EGFR [Watanabe, *et al.*, 1994]. However, previous studies have not assessed betacellulin binding to other erbB family receptors or betacellulin stimulation of erbB family receptors tyrosine phosphorylation or coupling to cellular signaling pathways.

Little is known about the in *vivo* functions of betacellulin. While its expression in the BTC-3 mouse insulinoma cell line [Shing, *et al.*, 1993] and the MCF-7 human breast adenocarcinoma cell line [Sasada, *et al.*, 1994] implies that betacellulin regulates the proliferation of pancreatic and breast cells and may play a causative role in breast and pancreatic cancer, the receptor(s) for betacellulin must be identified before definitive studies of the physiologic effects of betacellulin can be undertaken. In this study we have used derivatives of the Ba/F3 mouse pro-B lymphocyte cell line that have been engineered to ectopically express, singly and in pairwise combinations, all four erbB family receptors [Riese, *et al.*, in press]. We have assessed erbB family receptor tyrosine phosphorylation and the biological responses of these cell lines to stimulation with betacellulin and two other EGF family ligands, neuregulin-β (NRG-β) and EGF itself. Not only do these experiments identify a novel receptor for betacellulin, but we also demonstrate that betacellulin stimulates complex patterns of erbB family receptor tyrosine phosphorylation and coupling to cellular signaling pathways that are unique from the patterns stimulated by EGF and NRG-β.

Results

erbB family receptor tyrosine phosphorylation in single recombinant cell lines

The Ba/F3 cell line is a mouse pro-B lymphocyte cell line [Palacios and Steinmetz, 1985] that does not express endogenous EGFR, neu, and erbB-4, but does express low levels of erbB-3 [Riese, *et al.*, in press]. We have previously described the generation of Ba/F3 derivatives that ectopically express the four human erbB family receptors, singly and in all pairwise combinations [Riese, *et al.*, in press]. In this report, we have stimulated this panel of cell lines with recombinant human betacellulin, NRG-β, and EGF and assessed erbB family receptor tyrosine phosphorylation. The recombinant human betacellulin used in these experiments consisted of 34 amino acids of the human amphiregulin precursor (Val107 - Arg140), linked to the 50 amino acid EGF-structural motif of human betacellulin (Arg31 - Tyr80) and a 9 amino acid hemagglutinin epitope. The amphiregulin sequences in this molecule are not within the EGF-structural motif and therefore are not predicted to contribute to receptor binding. Furthermore, this recombinant betacellulin produced as a tagged fusion construct in bacteria had an activity indistinguishable from that produced transiently in COS cells using an expression construct containing the full length human betacellulin sequence (Plowman, *et al.*, in preparation).

In cell lines that ectopically express a single erbB family receptor, both betacellulin and EGF stimulated EGFR tyrosine phosphorylation (Figures 1 & 2a). Surprisingly, betacellulin (as well as NRG-β) stimulated erbB-4 tyrosine phosphorylation (Figures 1 & 2a). Not only do these data demonstrate that betacellulin regulates EGFR signaling, but they also indicate that erbB-4 is a receptor for betacellulin. Betacellulin did not stimulate increased neu or erbB-3 tyrosine phosphorylation (Figures 1 & 2a), suggesting that neither neu nor erbB-3 is a receptor for betacellulin (NRG-β stimulation of neu tyrosine phosphorylation is

probably due to endogenous erbB-3 expression [Riese, *et al.*, in press]). However, because erbB-3 has only minimal intrinsic tyrosine kinase activity [Guy, *et al.*, 1994], these data do not rule out the possibility that erbB-3 can bind betacellulin.

Since some AR sequences were present in the recombinant betacellulin used in these experiments, there was a remote possibility that these contributed to the unanticipated activation of erbB-4. Hence, we compared the activities of our betacellulin preparation with those of a recombinant mature-form preparation comprised of betacellulin exclusively (R&D Systems). In the cell line expressing erbB-4, both betacellulin preparations stimulated similar maximal levels of erbB-4 tyrosine at 3nM betacellulin (data not shown). This maximal level was similar to that stimulated by 6nM NRG-β (data not shown). Therefore, stimulation of EGFR and erbB-4 phosphorylation by betacellulin was not conferred by AR sequences.

erbB family receptor tyrosine phosphorylation in double recombinant cell lines

In activating both the EGFR and erbB-4, betacellulin displays activities distinct from EGF, which activates the EGFR alone, and NRG-β, which binds to erbB-3 and erbB-4. We next compared the effects of betacellulin, EGF, and NRG-β on receptor transmodulation by assessing receptor tyrosine phosphorylation in cell lines expressing combinations of erbB family receptors. Betacellulin, as well as EGF, stimulated the tyrosine phosphorylation of both receptors in the EGFR + neu (1+2), EGFR + erbB-3 (1+3), and EGFR + erbB-4 (1+4) cell lines (Figures 2b and 2c). Therefore, both betacellulin and EGF can transmodulate the other three receptors when co-expressed with EGFR. However, the three ligands did not stimulate equal levels of receptor phosphorylation. In 1+3 cells, all three ligands stimulated approximately equal levels of erbB-3 phosphorylation, while betacellulin and EGF stimulated higher levels of EGFR phosphorylation than NRG-β did (Figure 2b). Similarly, in 1+4 cells, betacellulin stimulated approximately equal levels of EGFR

and erbB-4 phosphorylation, while EGF stimulated higher levels of EGFR phosphorylation than erbB-4 phosphorylation and NRG-β stimulated higher levels of erbB-4 phosphorylation than EGFR phosphorylation (Figure 2c). One explanation for these differences is that EGFR, erbB-3, and erbB-4 may have a lower affinity for heterotypic interactions and transmodulation than for the homotypic interactions induced by direct stimulation.

As expected from the response of the erbB-4 cell line, betacellulin and NRG-β stimulated erbB-4 tyrosine phosphorylation in the three double recombinant cell lines that express erbB-4. However, while betacellulin and NRG-β stimulated EGFR and neu tyrosine phosphorylation in the EGFR + erbB-4 (1+4) and neu + erbB-4 (2+4) cell lines, respectively, betacellulin did not stimulate erbB-3 tyrosine phosphorylation in the erbB-3 + erbB-4 (3+4) cell line, even through NRG-β did (Figures 2c and 2d). Finally, betacellulin did not stimulate phosphorylation of either receptor in neu + erbB-3 cells, while NRG-β did (Figure 2c), suggesting that neither neu nor erbB-3 is a receptor for betacellulin.

IL-3-independent growth of Ba/F3 derivatives.

We next tested whether betacellulin, NRG-β, and EGF stimulate different patterns of receptor coupling to cellular signaling pathways. Since the recombinant Ba/F3 cell lines are strictly dependent upon interleukin-3 (IL-3) for survival and proliferation [Riese, *et al.*, in press], we tested whether betacellulin, NRG-β, or EGF induced the IL-3-independent survival or proliferation of the various Ba/F3 derivatives. Activation of either EGFR or neu in the single recombinant cell lines was associated with IL-3 independent survival but not proliferation (Figure 3a), while activation of erbB-3 or erbB-4 in the single recombinants had no biological effect (Figure 3a). Therefore, ligand stimulation of erbB phosphorylation was necessary, but not sufficient, for an IL-3 independent response (Table 1).

We also assessed ligand activity in the double recombinant Ba/F3 cell lines (Figure 3b). As expected from the responses of the single recombinant cell lines, receptor activation in cells expressing EGFR or neu conferred, with one notable exception, a minimal response of IL-3 independent survival. For example, in the EGFR + neu (1+2), EGFR + erbB-4 (1+4), neu + erbB-3 (2+3), and neu + erbB-4 (2+4) cell lines, receptor activation stimulated a minimum of IL-3-independent survival, while in erbB-3 + erbB-4 (3+4) cells none of the ligands stimulated an IL-3 independent response. The exception is the response of EGFR + erbB-3 (1+3) cells to ligand stimulation. As predicted, betacellulin and EGF stimulated the IL-3 independent survival of 1+3 cells; however, NRG-β failed to stimulate an IL-3 independent response, despite stimulating both EGFR and erbB-3 tyrosine phosphorylation in this cell line (Figure 2b).

Furthermore, in some of the double recombinant cell lines ligand stimulation of coupling of multiple receptors to signaling pathways acted in a non-additive manner to stimulate an IL-3 independent response (Figure 3b). In 1+2 cells betacellulin and EGF stimulated IL-3 independent proliferation, while in 1+4 cells betacellulin and NRG-β stimulated IL-3 independent proliferation and EGF stimulated a response intermediate to survival and proliferation. Therefore, while activation of either EGFR alone or neu alone stimulated IL-3 independent survival, in some cases activation of EGFR along with either neu or erbB-4 conferred IL-3 independent proliferation.

Discussion

Previous reports demonstrated that betacellulin binds to the A431 human adenocarcinoma cell line, which overexpresses the EGFR. This binding was quenched by the addition of an excess of EGF, suggesting that betacellulin is a ligand for the EGFR [Watanabe, *et al.*, 1994]. However, the effect of betacellulin on EGFR tyrosine phosphorylation and signaling and the possibility that betacellulin might activate other erbB family receptors were not assessed. Here we show that in Ba/F3 cells expressing only a single ectopic erbB family receptor, betacellulin stimulates the tyrosine phosphorylation of both the EGFR and, surprisingly, erbB-4 (Table 1). This is consistent with the observation that radiolabeled betacellulin binds specifically to EGFR and erbB-4, but not to neu (Plowman, *et al.*, in preparation). Thus, betacellulin exhibits activities that are distinct from those displayed by EGF, which activates the EGFR alone, and NRG-β, which activates erbB-3 and erbB-4 (Table 1). Furthermore, in this first comprehensive analysis of erbB family transmodulation for both betacellulin and EGF, we find that EGF can transmodulate erbB-4 in the EGFR + erbB-4 cell line (Table 1). We also demonstrate that betacellulin stimulates a pattern of receptor transmodulation that is qualitatively distinct from the patterns stimulated by EGF and NRG-β.

With one exception, betacellulin, EGF, and NRG-β transmodulated the tyrosine phosphorylation of all four erbB family receptors in cell lines that express any receptor for each ligand (Table 1). Thus, differences in ligand activities in the double recombinant cell lines can be predicted by differences in activities in the single recombinant cell lines. For example, NRG-β activates erbB-3, while betacellulin does not activate neu or erbB-3. Not surprisingly, in cells expressing neu + erbB-3, NRG-β stimulates the phosphorylation of both receptors, while betacellulin does not stimulate the phosphorylation of either receptor in this cell line (Table 1). The single exception is the response of the erbB-3 + erbB-4 (3+4) cell

line to betacellulin. Both betacellulin and NRG-β stimulate erbB-4 tyrosine phosphorylation in the single recombinant cell line. However, in 3+4 cells, NRG-β stimulates the tyrosine phosphorylation of both receptors, while betacellulin stimulates the tyrosine phosphorylation of erbB-4 but not of erbB-3 (Table 1). Nonetheless, because NRG-β binds erbB-3, it is possible that this absence of erbB-3 tyrosine phosphorylation may not be due to differences between betacellulin- or NRG-β-induced erbB-3 transmodulation.

Previous work demonstrated that different erbB family receptors or combinations of receptors couple to distinct cellular signaling pathways. For example, EGFR activation stimulates the tyrosine phosphorylation of four proteins that are not highly phosphorylated following neu activation [Fazioli, *et al.*, 1992]. Furthermore, activated erbB-3 stimulated higher levels of phosphatidylinositol 3-kinase than EGFR did [Fedi, *et al.*, 1994; Soltoff, *et al.*, 1994; Carraway, *et al.*, 1995], and it has been suggested that EGFR and neu bind the adapter protein GRB2, but erbB-3 does not [Prigent and Gullick, 1994; but also see Kim, *et al.*, 1994; Fedi, *et al.*, 1994]. These different coupling capacities of the erbB family receptors can be correlated to specific biological responses. Activation of the EGFR stimulates the IL-3 independent proliferation of 32D myeloid cells, while wild-type and mutationally-activated neu alleles do not [DiFiore, *et al.*, 1990]. In Ba/F3 cells, however, activation of neu stimulates IL-3 independent survival, while activation of EGFR and erbB-4 together stimulates IL-3 independent proliferation [Riese, *et al.*, in press].

We found that betacellulin stimulates IL-3 independent survival or proliferation in neu + erbB-4 cells and in every cell line that expresses EGFR. In contrast, EGF stimulated IL-3 independence only in those cell lines that express EGFR while NRG-β stimulated IL-3 independence only in the EGFR + erbB-4 cell line and in those cell lines that express neu (Table 1). Therefore, with a single exception, the minimal requirement for IL-3 independence is activation of either

EGFR or neu. The exception is that betacellulin and EGF, but not NRG-β, stimulated IL-3 independent survival in the EGFR + erbB-3 cell line (Table 1). This lack of response to NRG-β may merely reflect the lower level of EGFR phosphorylation stimulated by NRG-β in this cell line (Figure 2b). On the other hand, the absence of biological response may reflect the different mechanism by which the EGFR is activated. We demonstrated previously that coupling of multiple receptor species to cellular signaling pathways acts in a non-additive manner in specifying biological responses [Riese, *et al.*, in press]. Accordingly, while activation of EGFR or neu by themselves stimulated IL-3 independent survival, activation of EGFR and either neu or erbB-4 together stimulated IL-3 independent proliferation (Table 1).

Biological responses to EGF family ligands are regulated by several hierarchical mechanisms [Riese, *et al.*, in press]. Some, but not all, of these mechanisms are shared by other networks of receptor tyrosine kinases and their ligands, including the neurotrophin network and the fibroblast growth factor (FGF) network. Like the EGF family, the neurotrophin and FGF ligand families have several members that can each activate multiple receptors. The neurotrophin ligand family includes nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4, also known as NT-4/5 or NT-5 [Reviewed in Barbacid, 1994], while the FGF family has at least 9 members encoded by different genes [Reviewed in Johnson and Williams, 1994]. Furthermore, like the erbB receptor family, both the FGF receptor and neurotrophin receptor families have multiple members (FGFR-1, FGFR-1, FGFR-3, FGFR-4 and TrkA, TrkB, TrkC, respectively). Moreover, like the EGFR and erbB-4, some of these FGFRs and Trks can bind multiple ligands [Reviewed in Johnson and Williams, 1994; Barbacid, 1994].

Another regulatory mechanism common to the EGF/erbB and FGF signaling networks is that both use heparan sulfate proteoglycans (HSPGs) to modulate

receptor-ligand interactions. FGFs bind with low affinity in a multivalent manner to HSPGs, causing ligand oligomerization [Reviewed in Lemmon and Schlessinger, 1994] and increasing their binding affinity for FGFRs [Reviewed in Eckenstein, 1994]. Because the FGF/FGFR complex exists in a 1:1 stoichiometry [Spivak-Kroizman, *et al.*, 1994], yet FGFs are monomeric, it has been proposed that HSPG binding potentiates FGF stimulation of FGFR phosphorylation and dimerization. HSPGs also regulate the interactions of EGF family ligands with their receptors. Several EGF family ligands bind HSPGs, including NRGs, amphiregulin (AR), and heparin-binding-EGF-like growth factor (HB-EGF), and this binding regulates ligand-receptor interactions [Aviezer and Yayon, 1994; Johnson and Wong, 1994; Cook, *et al.*, 1995a; Cook, *et al.*, 1995b]. However, many of the mechanistic details of regulation by HSPGs have yet to be elucidated.

While the neurotrophin and FGF networks have regulatory mechanisms that are also features of the EGF/erbB network, there are also features of the neurotrophin and FGF networks that are not properties of the EGF/erbB network. Alternative splicing produces truncated FGFR and Trk isoforms lacking the cytoplasmic tyrosine kinase domain and sites for tyrosine phosphorylation [Reviewed in Johnson and Williams, 1994; Barbacid, 1994. Therefore, a regulatory mechanism not observed in the EGF/erbB network results in dominant negative receptors, which are not a characteristic of the EGF/erbB network. Another feature that is characteristic of the neurotrophin network and not seen in the EGF/erbB network is regulation by a low-affinity co-receptor. p75, the low-affinity neurotrophin receptor, has no tyrosine kinase domain [Reviewed in Chao, 1994] and p75 binding is in some cases dispensable for biological response [Reviewed in Ibanez, 1994]. Nonetheless, it has been proposed that p75 regulates the biological response to neurotrophins by altering the affinity of neurotrophin binding to the Trk family receptors [Benedetti, *et al.*, 1993; Reviewed in Chao, 1994].

Data presented here suggests that differences in NRG-β, EGF, and betacellulin activities play a significant role in specifying the proliferation and differentiation of human tissues *in vivo*. These ligands may play their most significant role in the mammary epithelium and tissues of neuroectodermal origin. Not only has the expression of EGF family ligands been documented in these cell types, but these ligands can regulate the proliferation and/or differentiation of these cell types in cultured cell or animal model systems. Furthermore, mounting evidence suggests that increases in the expression and/or signaling of erbB family receptors plays a significant role in tumors of mammary or neuroectodermal origin [Reviewed in Hynes and Stern, 1994]. Because betacellulin, NRG-β, and EGF have distinct biological activities that apparently reflect their differing abilities to activate receptor signaling, it may be possible to develop antagonists that specifically disrupt signaling by a single EGF family ligand and may inhibit the genesis or growth of malignancies without disrupting the activity of other EGF family ligands in the same tissue.

Materials and Methods

Production of human recombinant Betacellulin

To facilitate refolding and purification, human recombinant betacellulin was produced as an epitope-tagged fusion with human amphiregulin. The pPL-Lambda (Pharmacia) thermoinducible bacterial expression vector was modified to remove the EcoRI, BamHI, and SmaI sites upstream from the PL promoter and a human betacellulin transcription unit was inserted into the unique HpaI site within the N gene. The insertion contained the lac and Cro gene Shine-Delgarno ribosome binding sites; a unique BglII cloning site; an initiating methionine codon; the nucleotide sequence encoding 34 amino acids of the human amphiregulin precursor (Val107 - Arg140), the 50 amino acid EGF-structural motif of human betacellulin (Arg31 - Tyr80), and a 9 amino acid hemagglutinin epitope sequence (PYDVPDYAS); a stop codon; unique EcoRV and XbaI restriction sites; and transcription termination sequences. The resulting plasmid, pPLABTC-Tag, was transformed into competent E. Coli N4830-1 and grown at 30°C in 1 liter LB media with 50 ug/ml ampicillin to an Abs_{600} of 0.7. Cultures were then induced by incubation at 42°C for 18-24 hr. Following induction, cells were harvested by centrifugation at 5000xg, washed in STE buffer (50 mM Tris, pH 8.0/200 mM NaCl/2 mM EDTA). The pellet was resuspended in STE containing 2 mM 2-mercaptoethanol, and lysed by addition of 0.2 mg/ml lysozyme followed by addition of Triton X-100 and Zwittergent (CalBiochem) to 1%. To ensure lysis and solubilization of non-inclusion body protein, the preparation was sonicated for 2 min, and centrifuged at 13000xg for 10 min. The pellet was resuspended in STE and sonicated again for an additional 1 min. The slurry was then layered on a 40% sucrose cushion and centrifuged at 13000xg for 10 minutes at 4°C. The inclusion body pellet was resuspended in 6 M guanidine-HCl (GuHCL)/50 mM CAPS, pH 11.0.

The Betacellulin inclusion body preparation was diluted to 60 mM GuHCl with 50 mM CAPS, pH 11.0/1 mM EDTA/1.25 mM reducing glutathione/0.5 mM oxidizing glutathione. The final protein concentration was 50-100 mg/ml by Biorad protein assay. Refolding was achieved by incubation at 4°C for 18-24 hr. The solution was then dialyzed against 50 mM sodium phosphate (NaP), pH 7.5, and successively filtered through 5 mm, 0.45 mm, and 0.22 mm filters or subjected to 60,000xg centrifugation prior to cation exchange chromatography. Alternatively, the refolded material was buffer exchanged by ultrafiltration against 3 volumes of 50 mM NaP, pH 7.5.

Cleared, refolded bacterially-produced betacellulin was loaded on a cation exchange column (Bakerbond CSx) equilibrated with 40 mM NaP pH 7.0. The flow rate was 1.25 ml/min and the chromatography was carried out at room temperature. The column was washed with 20 column volumes of 40 mM NaP, pH 7.0, or until a stable baseline was achieved. The betacellulin was eluted with a 50 ml linear gradient of 0.2 -1 M NaCl in 40 mM NaP, pH 7.0. The peak fractions were at ~550 mM NaCl as determined by reactivity in a hemagluttinin ELISA. The peak fractions from cation exchange chromatography were pooled, diluted to 0.2 M NaCl with 40 mM NaP, pH 7.0, and applied to an FPLC TSK-heparin 5PW column (TosoHaas). The flow rate was 1 ml/min. The column was then washed with 40 mM NaP, pH 7.0 and bound protein was eluted with a 30 ml linear gradient of 0 - 1.0 M NaCl in 40 mM NaP, pH 7.0. The recombinant tagged betacellulin eluted at ~800 mM NaCl, and migrated as a single Coomassie stained band on 15% SDS-PAGE.

Betacellulin activity was measured using an EGFR tyrosine phosphorylation assay (Thorne and Plowman, 1994). Recombinant betacellulin produced as a tagged fusion construct in bacteria had an activity indistinguishable from that produced transiently in COS cells using an expression construct containing the full length human betacellulin sequence (Plowman, *et al.*, in preparation).

Cell lines and cell culture

cDNAs encoding the four human erbB family receptors were subcloned into the recombinant retroviral expression vector pLXSN [Miller and Rosman, 1989], which carries a neomycin resistance gene. pLXSN and the constructs expressing the erbB family receptors were transfected into the mouse Ba/F3 pro-B-lymphocyte cell line [Palacios and Steinmetz, 1985] and selected with geneticin, generating a vector control cell line as well as clonal cell lines that express the four erbB family receptors, singly and in pairwise combinations. The generation of these cell lines has been described previously [Riese, *et al.*, in press]. The cell lines used in this report are: LXSN/1 (vector control); EGFR/3; neu/12C; erbB-3/3; erbB-4/7; EGFR+neu/5D; EGFR+erbB-3/4A; EGFR+erbB-4/2A; neu+erbB-3/7A; neu+erbB-4/15A; and erbB-3+erbB-4/2B. Cells were grown in RPMI (Gibco/BRL) supplemented with 10% fetal calf serum (Sigma), 200ug/ml G418 (Gibco/BRL), and interleukin-3 (IL-3) supplied as 10% conditioned medium from the WEHI-3B mouse myelomonocytic leukemia cell line [Daley and Baltimore, 1988].

The ranked order of receptor expression in the double recombinant cell lines is as follows. For EGFR expression, EGFR + erbB-4/2A is higher than EGFR + neu/5D, which is higher than EGFR + erbB-3/4A. For neu expression, neu + erbB-4/15A is marginally higher than EGFR + neu/5D, which is markedly higher than neu + erbB-3/7A. The levels of ErbB-3 expression are similar in the EGFR + erbB-3/4A, neu + erbB-3/7A, and erbB-3 + erbB-4/2B cell lines, while the levels of erbB-4 expression are similar in the EGFR + erbB-4/2A, neu + erbB-4/15A and erbB-3 + erbB-4/2B cell lines [Riese, *et al.*, in press].

Stimulation and analysis of erbB family receptor tyrosine phosphorylation

$2x10^8$ recombinant Ba/F3 cells were washed in phosphate-buffered saline (PBS) and resuspended in 50ml RPMI supplemented with IL-3. The cells were incubated for 6-10 hours at 37°C, washed in ice-cold PBS, and resuspended in 1-3ml ice-cold PBS. Remaining steps were performed cold or on ice. The cells were transferred in two to five 0.5ml portions to microcentrifuge tubes. Human recombinant EGF (Collaborative Biomedical), a chemically-synthesized NRG-β 65-mer [Barbacci, *et al.*, 1995; Riese, *et al.*, in press] or recombinant human betacellulin was added at a final concentration of 100ng/ml (EGF), 94ng/ml (NRG-β), or 150ng/ml (betacellulin). Following a 10-minute incubation, cells were lysed and the protein content of each sample was assayed as described earlier [Riese, *et al.*, in press].

Analysis of erbB family receptor tyrosine phosphorylation has been described previously [Riese, *et al.*, in press]. Briefly, the erbB family receptors were immunoprecipitated with antisera specific for single receptors, after which the samples were separated by electrophoresis through a 7.5% acrylamide, 0.17% bisacrylamide, 0.1% SDS gel [Sefton, *et al.*, 1979], electrotransferred onto nitrocellulose [DiGiovanna and Stern, 1995], and immunoblotted with the antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.). Antibody binding was detected with sheep anti-mouse coupled to horseradish peroxidase antibody NA931 (Amersham) and enhanced chemiluminescence reagents RPN2106 (Amersham).

Anti-receptor antibodies used for immunoprecipitation were as follows: anti-EGFR mouse monoclonal antibody 528 [Gill, *et al.*, 1984]; anti-Neu mouse monoclonal antibodies TAb 250 [Langton, *et al.*, 1991], FSP16 [Harwerth, *et al.*, 1992], and TA-1 (Ab-5, Oncogene Science); anti-erbB-3 rabbit polyclonal antiserum SC-285 (Santa Cruz Biotechnology); and anti-erbB-4 rabbit polyclonal antiserum SC-283

(Santa Cruz Biotechnology). Specificity of anti-receptor antibodies has been verified previously by testing for cross-reactivity with lysates from cell lines expressing heterologous receptors (data not shown).

Stimulation and analysis of IL-3 independence

For each trial and treatment, Ba/F3 derivatives made quiescent by growth to saturation density were plated at a density of $100x10^3$ cells/ml in duplicate culture dishes containing medium lacking IL-3 (IL-3-Free), medium supplemented with IL-3 (IL-3), or in medium lacking IL-3 but supplemented with 10 ng/ml human recombinant EGF (EGF - Collaborative Biomedical), 9.4ng/ml chemically-synthesized NRG-β 65- mer (Neuregulin - [Barbacci, *et al.*, 1995; Riese, *et al.*, in press]), or 7ng/ml human recombinant betacellulin (Betacellulin). Cells were stained daily with trypan blue and counted in a hemacytometer to determine viable cell densities until each sample reached a viable cell saturation density, at which time data collection was terminated. While not every cell line was tested with every factor in every trial, each combination of cell lines and factors shown was tested in a minimum of 4 trials, while some combinations were tested in as many as 20 trials. The arithmetic means and the standard error of the means of the viable cell saturation densities were calculated for each combination of cell line and treatment [Zar, 1984]. Cultures exhibiting viable cell saturation densities of less than $20x10^3$ cells/ml were judged to be nonresponsive to stimulation. Cultures with viable cell saturation densities of $50\text{-}400x10^3$ cells/ml were judged to be exhibiting survival but not proliferation. Cultures with viable cell saturation densities of greater than $800x10^3$ cells/ml were judged to be exhibiting proliferation.

Acknowledgments

The authors thank Brad Guarino, Glenn C. Andrews, and James C. Moyer, Pfizer Central Research, for providing the synthetic NRG-β. We thank John Koland, Nancy Hynes, and Hideo Masui for the generous gifts of anti-erbB antibodies. We also thank Edward Kim, Michael DiGiovanna, Debbie Hayden, and other members of the Stern laboratory for their advice, reagents, and technical support. This work was supported by Public Health Service grant CA-45708 from the National Cancer Institute and by grant DAMD-17-94-J-4476 from the United States Army Medical Research and Materiel Command to D.F.S. D.J.R. was supported by Public Health Service post-doctoral training grant HD-07149 from the National Cancer Institute and by postdoctoral fellowship DAMD-17-94-J-4036 from the United States Army Medical Research and Materiel Command. T.M.v.R. was supported by a Dutch Cancer Society fellowship. The content of the information contained herein does not necessarily reflect the position or policy of the United States Government, and no official endorsement should be inferred.

References

Akiyama T, et al. (1988). *Mol. Cell. Biol.* **8**, 1019-1026.

Aviezer D and Yayon A. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 12173-12177.

Barbacci EG, *et al.* (1995). *J. Biol. Chem.* **270**, 9585-9589.

Barbacid M. (1994). *J. Neuobiology* **25**, 1386-1403.

Benedetti M, Levi A, and Chao MV. (1993). *Proc. Natl. Acad. Sci. USA* **90**, 7859-7863.

Carraway KL III, *et al.* (1994). *J. Biol. Chem.* **269**, 14303-14306.

Carraway KL III, Soltoff SP, Diamonti AJ, and Cantley LC. (1995). *J. Biol. Chem.* **270**, 7111-7116.

Chao MV. (1994). *J. Neuobiology* **25**, 1373-1385.

Connelly PA and Stern DF. (1990), *Proc. Natl. Acad. Sci. USA* **87**, 6054-6057.

Cook PW, *et al.* (1995a). *J. Cell. Phys.* **163**, 407-417.

Cook PW, *et al.* (1995b). *J. Cell. Phys.* **163**, 418-429.

Daley GQ and Baltimore D. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 9312-9316.

DiFiore PP, *et al.* (1990). *Science* **248**, 79-83.

DiGiovanna MP, and Stern DF. (1995). *Cancer Res*, **55**, 1946-1955.

Earp HS, Dawson TL, Xiong L, and Hong Y. (1995). *Breast Cancer Res. Treat.* **35**, 115-132.

Eckenstein FP. (1994). *J. Neurobiology* **25**, 1467-1480.

Fazioli F, et al. (1992). *J. Biol. Chem.* **267**, 5155-5161.

Fedi P, Pierce JH, DiFiore PP, and Kraus MH. (1994). *Mol. Cell. Biol.* **14**, 492-500.

Gill GN, *et al.* (1984). *J. Biol. Chem.* **259**, 7755-7760.

Goldman R, Ben Levy R, Peles E, and Yarden Y. (1990). *Biochemistry*, **29**, 11024-11028.

Groenen LC, Nice EC, and Burgess AW. (1994). *Growth Factors* **11**, 235-257.

Guy PM, *et al.* (1994). *Proc. Natl. Acad. Sci. USA* **91**, 8132-8136.

Harwerth IM, Wels W, Marte BM, and Hynes NE. (1992). *J. Biol. Chem.* **267**, 15160-15167.

Hynes NE and Stern DF. (1994). *Biochimica et Biophysica Acta*, **1198**, 165-184.

Ibanez CF. (1994). *J. Neuobiology* **25**, 1349-1361.

Ip NY, *et al.* (1993). *Neuron* **10**, 137-149.

Johnson DE and Williams LT. (1993). *Adv. Cancer Res.* **60**, 1-41.

Johnson GR, Kannan B, Shoyab M, and Stromberg K. (1993). *J. Biol. Chem.* **268**, 2924-2931.

Johnson GR and Wong L. (1994). *J. Biol. Chem.* **269**, 27149-27154.

Karunagaran D, *et al.* (1995). *J. Biol. Chem.* **270**, 9982-9990.

Kim HH, Sierke SL, and Koland JG. (1994). *J. Biol. Chem.* **269**, 24747-24755.

King CR, *et al.* (1988). *EMBO J.* **7**, 1647-1651.

Kita YA, et al. (1994). *FEBS Lett.* **349**, 139-143.

Langton BC, *et al.*, (1991). *Cancer Res.* **51**, 2593-2598.

Lemmon MA and Schlessinger J. (1994) *Trends Biol. Sci.* **19**, 459-463.

Miller AD and Rosman GJ. (1989). *BioTechniques* **7**, 980-990.

Palacios R and Steinmetz M. (1985). *Cell* **41**, 727-734.

Plowman GD, *et al.* (1993). *Nature* **366**, 473-475.

Prigent SA and Gullick WJ. (1994). *EMBO J.* **13**, 2831-2841.

Qian X, *et al.* (1992). *Proc. Natl. Acad. Sci. USA* **89**, 1330-1334.

Riese DJ II, *et al. Mol. Cell. Biol.*, in press.

Sasada R, *et al.* (1993). *Biochem. Biophys. Res. Com.*, **190**, 1173-1179.

Sefton B, Beemon K, and Hunter T. (1979). *J. Virol.* **28**, 957-971.

Shing Y, *et al.* (1993). *Science* **259**, 1604-1607.

Sliwkowski MX, *et al.* (1994). *J. Biol. Chem.* **269**, 14661-14665.

Soltoff SP, *et al.* (1994). *Mol. Cell. Biol.* **14**, 3550-3558.

Spivak-Kroizman T, *et al.* (1992). *J. Biol. Chem.* **267**, 8056-8063.

Spivak-Kroizman T, *et al.* (1994). *Cell* **79**, 1015-1024.

Stern DF and Kamps MP. (1988). *EMBO J.*, **7**, 995-1001.

Thorne BA and Plowman GD. (1994) *Mol. Cell. Biol.* **14**, 1635-1646.

Ueno H, *et al.* (1992). *J. Biol. Chem.* **267**, 1470-1476.

Wada T, Qian X, and Greene MI. (1990). *Cell*, **61**, 1339-1347.

Watanabe T, *et al.* (1994). *J. Biol. Chem.* **269**, 9966-9973.

Zar JH. (1984). *Biostatistical Analysis, 2nd ed.* Prentice-Hall, Inc., Englewood Cliffs, N.J.

Table 1. Summary of Stimulation of Receptor Tyrosine Phosphorylation and IL-3 Independence

Cell Line	Receptor	Betacellulin Tyr Phos.[a]	Betacellulin IL-3 Indpt.[b]	NRG-β Tyr Phos.[a]	NRG-β IL-3 Indpt.[b]	EGF Tyr Phos.[a]	EGF IL-3 Indpt.[b]
EGFR		+	S	-	N	+	S
Neu		-	N	+[c]	S[c]	-	N
erbB-3		-	N	-	N	-	NT[d]
erbB-4		+	N	+	N	-	NT[d]
EGFR+Neu	EGFR	+	P	*[c]	S[c]	+	P
	Neu	+		*[c]		+	
EGFR+erbB-3	EGFR	+	S	+	N	+	S
	erbB-3	+		+		+	
EGFR+erbB-4	EGFR	+	P	+	P	+	S/P
	erbB-4	+		+		+	
Neu+erbB-3	Neu	-	N	+	S	-	NT[d]
	erbB-3	-		+		-	
Neu+erbB-4	Neu	+	S	+	S	-	N
	erbB-4	+		+		-	
erbB-3+erbB-4	erbB-3	-	N	+	N	-	N
	erbB-4	+		+		-	

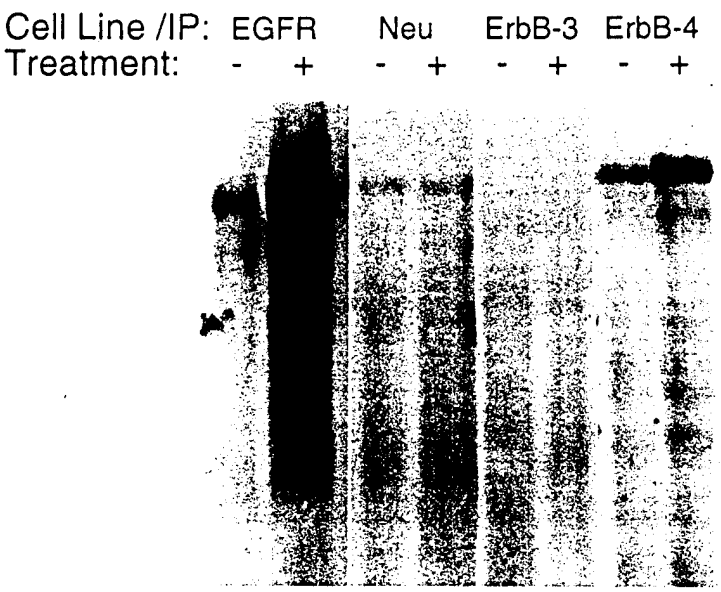
[a]Results are abstracted from Figures 1 and 2, Riese, *et al.*, in press, and similar unpublished data. "+" indicates increased receptor tyrosine phosphorylation over basal levels, "-" indicates no increase in receptor tyrosine phosphorylation, and "*" indicates ambiguity due to high basal levels of receptor phosphorylation.

[b]Results are abstracted from Figure 3. "N" indicates no IL-3 independent response, "S" indicates stimulation of IL-3 independent survival, "P" indicates stimulation of IL-3 independent proliferation, and "S/P" indicates stimulation of an intermediate response. "NT" indicates not tested.

[c]The NRG response is apparently due to interactions with the endogenous erbB-3 in Ba/F3 cells [Riese, *et al.*, in press].

[d]Given the absence of receptor tyrosine phosphorylation, no IL-3 independent response is expected.

Fig 1

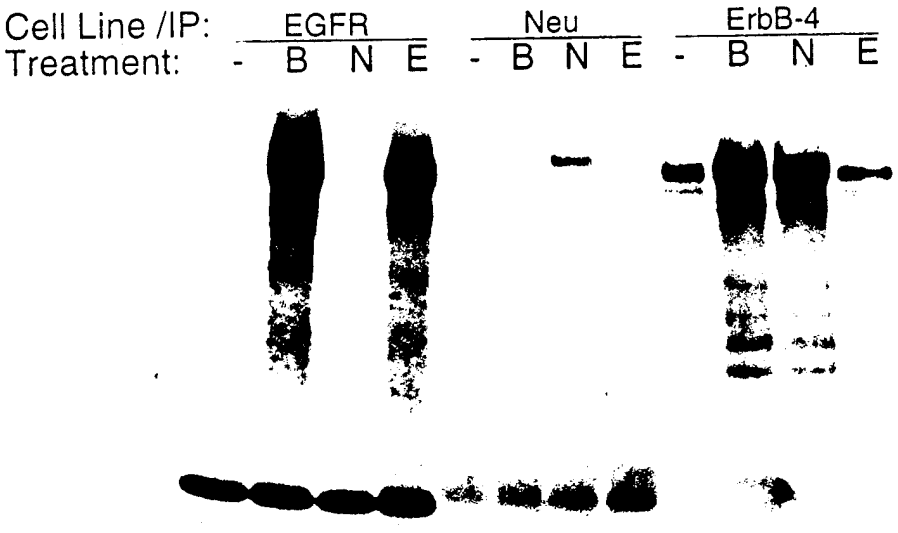


Figures and Figure Legends

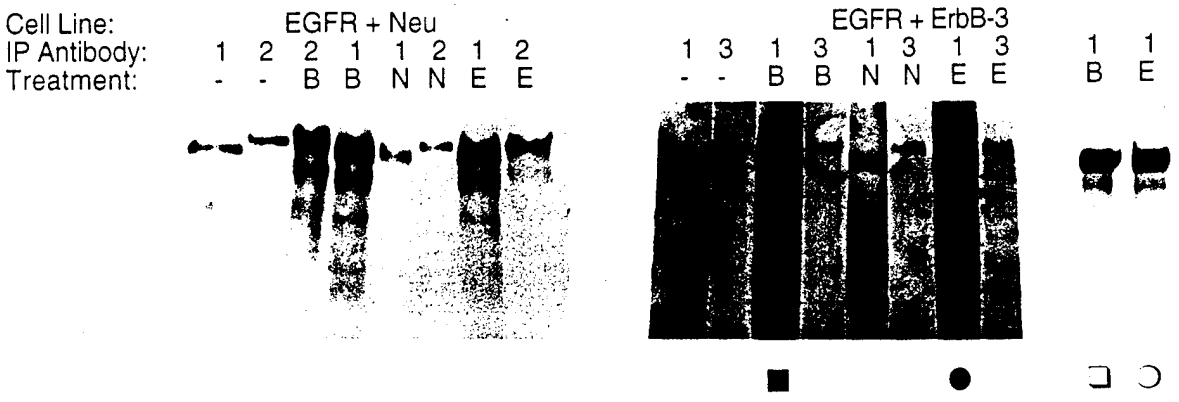
Figure 1. Regulation of receptor tyrosine phosphorylation by betacellulin in single recombinant Ba/F3 derivatives.

Lysates from untreated or betacellulin-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody. The cell lines and immunoprecipitating antibodies are as marked. Lysates from betacellulin-treated cells are denoted "+" while lysates from untreated cells are denoted "-".

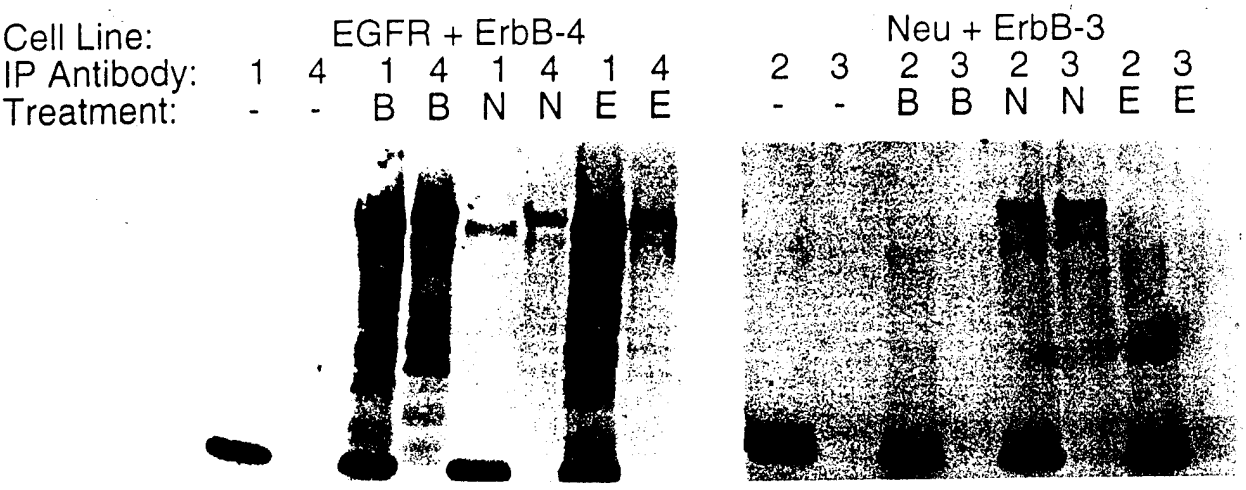
2a


Cell Line /IP:
EGFR
Neu
ErbB-4
Treatment:
- B N E - B N E - B N E

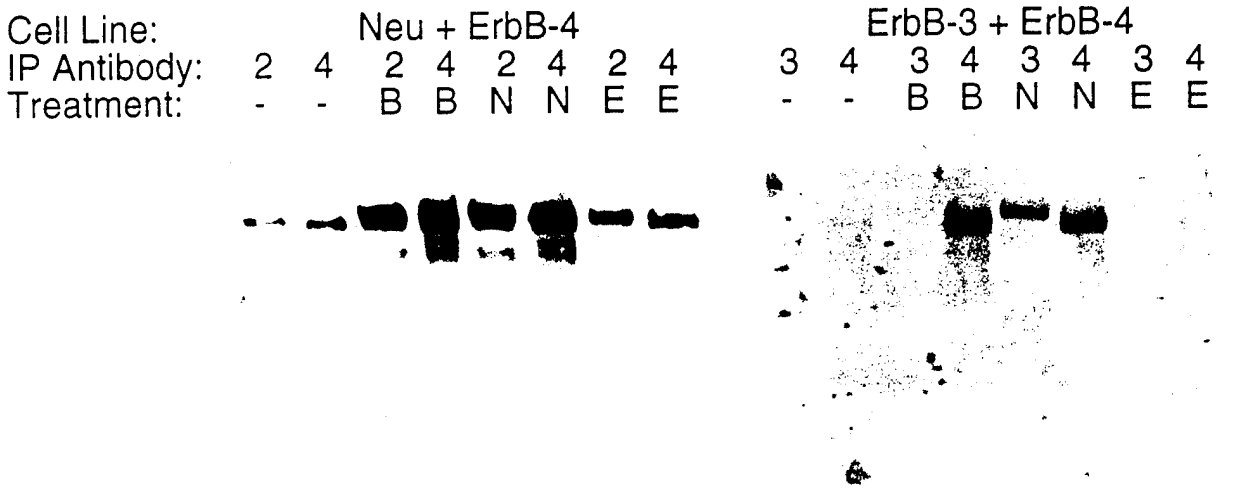
2b


Cell Line:
EGFR + Neu
EGFR + ErbB-3
IP Antibody:
1 2 2 1 1 2 1 2
1 3 1 3 1 3 1 3
1 1
Treatment:
- - B B N N E E
- - B B N N E E
B E

2c


Cell Line:
EGFR + ErbB-4
Neu + ErbB-3
IP Antibody: 1 4 1 4 1 4 1 4
2 3 2 3 2 3 2 3
Treatment: - - B B N N E E
- - B B N N E E

2d

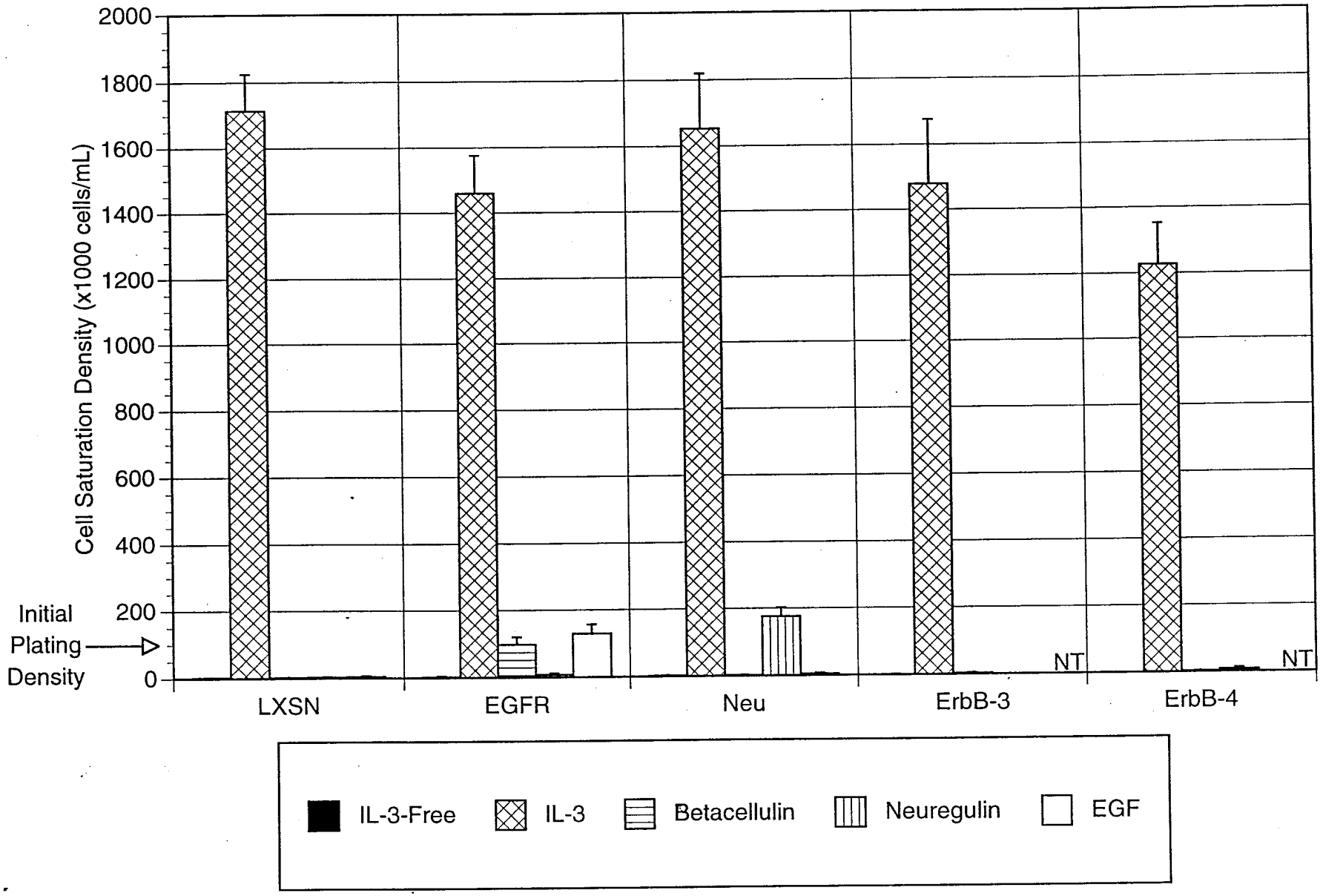

Cell Line:
Neu + ErbB-4
ErbB-3 + ErbB-4
IP Antibody: 2 4 2 4 2 4 2 4
3 4 3 4 3 4 3 4
Treatment: - - B B N N E E
- - B B N N E E

Figures 2a-d. Comparison of receptor tyrosine phosphorylation in Ba/F3 derivatives stimulated with betacellulin, EGF, or NRG-β.

Lysates from untreated or ligand-treated Ba/F3 derivatives were immunoprecipitated with anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibody. The cell lines are as marked. Immunoprecipitating antibodies were: α1 or 1, anti-EGFR; α2 or 2, anti-Neu; α3 or 3, anti-erbB-3; α4 or 4, anti-erbB-4. Lysates from betacellulin-treated cells are denoted "B", lysates from NRG-β-treated cells are denoted "N", lysates from EGF-treated cells are denoted "E", and lysates from untreated cells are denoted "-". Lighter exposures of the lanes denoted by ■ and ● are denoted by ❑ and ○.

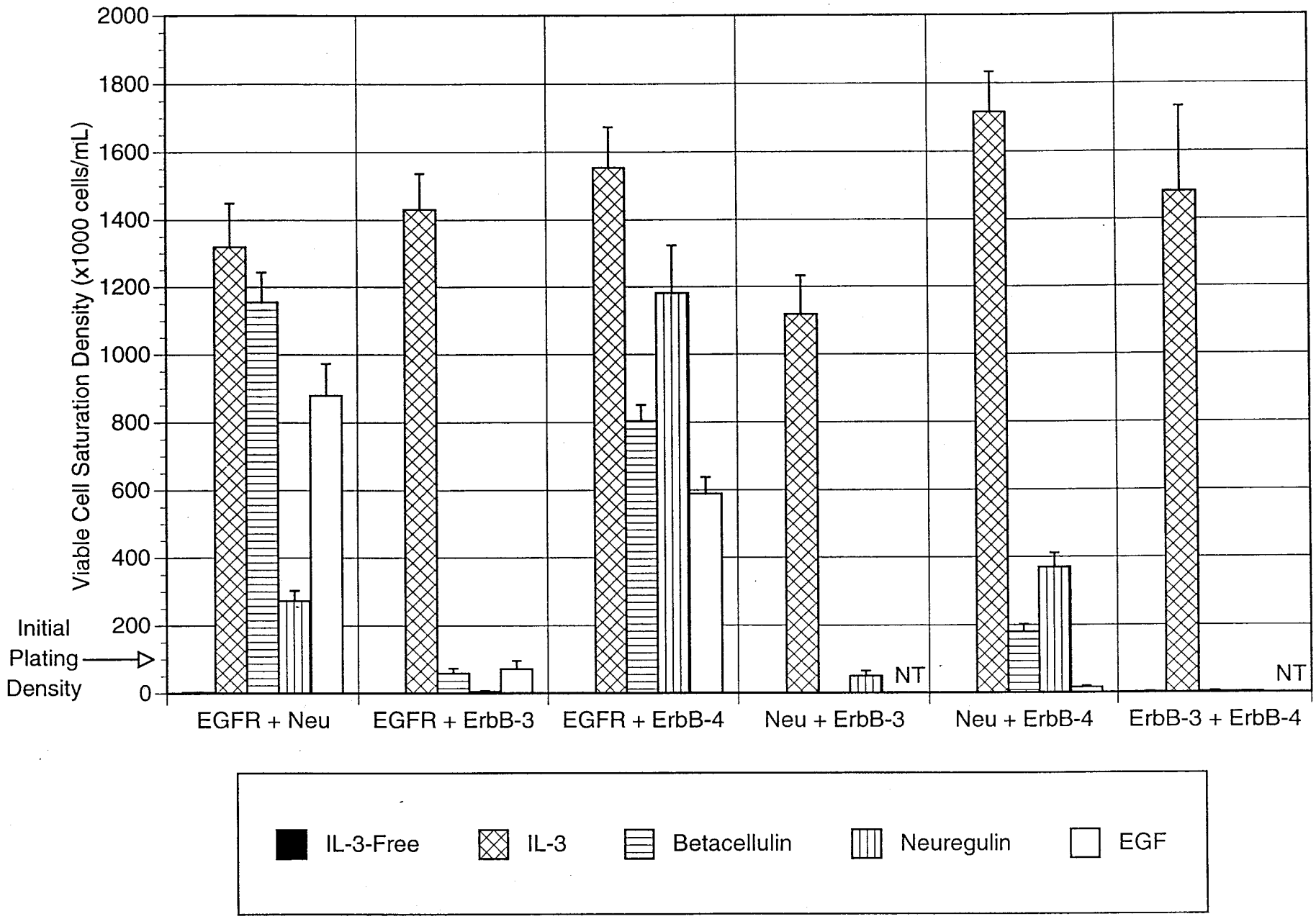
3a

IL-3 Independence Assay



3b

IL-3 Independence Assay



neuregulins

Figures 3a-b. IL-3-independent saturation density of Ba/F3 cells treated with betacellulin, EGF, or NRG-β.

Ba/F3 derivatives were plated at a density of $100x10^3$ cells/ml in medium lacking IL-3 (IL-3-Free), medium supplemented with IL-3 (IL-3), or in medium lacking IL-3 but supplemented with 10 ng/ml human recombinant EGF (EGF), 9.4ng/ml NRG-β 65- mer (Neuregulin), or 7ng/ml human recombinant betacellulin (Betacellulin). Viable cell saturation densities were calculated two to four days after seeding. Each combination of cell lines and factors was tested four to twenty times, and the arithmetic means of the viable cell saturation densities are indicated by the filled bars, while the standard error of the means are indicated by the error bars. Because cultures treated with IL3-free medium exhibited densities of less than $4x10^3$ cells/ml, these values, represented by the left-most bar for each cell line, are not apparent on the graphs. Some of the data describing the responses to stimulation with NRG-β or control media has been previously published [Riese, *et al.*, in press]. "NT" indicates not tested.